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(71) Applicant *(for all designated States except US)*: **NASTECH PHARMACEUTICAL COMPANY INC.** [US/US]; 3830 Monte Villa Parkway, Bothell, WA 98021-7266 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **QUAY, Steven, C.** [US/US]; PMB 421, 14241 N.E., Woodinville-Duvall Road,, Woodinville, WA 98072 (US). **JOHNSON, Paul, Hickok** [US/US]; 12020 211th Place Southeast, Snohomish, WA 98296 (US). **HOUSTON, Michael, E., Jr.** [CA/US]; 2818 220 Place Northeast, Sammamish, WA 98074 (US). **CUI, Kunyuan** [US/US]; 3224-189th Street Southeast, Bothell, WA 98012 (US). **AHMADIAN, Mohammad** [IR/US]; 3106 200th Place Southeast, Bothell, WA 98012 (US). **CHEN, Lishan** [US/US]; 13620 Southeast 43rd Place, Bellevue, WA 98006 (US). **CHEN, Yuching** [US/US]; 13620 Southeast 43rd Place, Bellevue, WA 98006 (US). **MAYER, Sasha, J.** [CA/US]; 6011 174th Street Southeast, Snohomish, WA 98296 (US). **FAM, Renata** [PL/US]; 1901 Northeast 85th Street, Seattle, WA 98115 (US).

(74) Agents: **KNUDSEN, Peter, J.** et al.; Nastech Pharmaceutical Company Inc., 3830 Monte Villa Parkway, Bothell, WA 98021-7266 (US).

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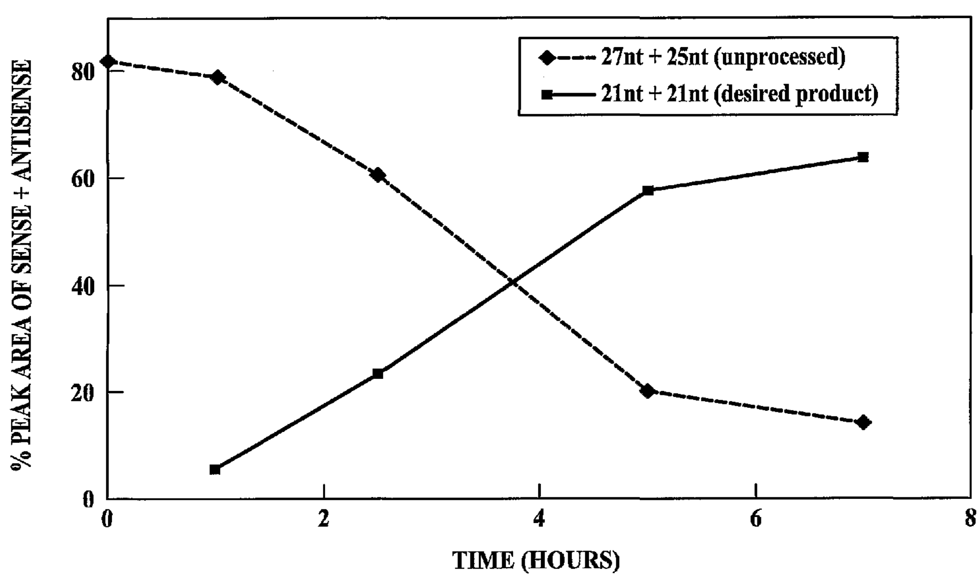
(54) Title: PEPTIDE-DICER SUBSTRATE RNA CONJUGATES AS DELIVERY VEHICLES FOR SIRNA



(57) Abstract: Provided are compositions comprising a double stranded ribonucleic acid (dsRNA) molecule and a peptide of about 5 to about 40 amino acids, wherein the dsRNA molecule is conjugated to the peptide. The strands of the dsRNA may have lengths from about 25 to about 30 base pairs, which may be the same or different. siRNA may, alternatively, comprise at least three strands (i.e., either at least two sense strands and one antisense strand or at least two antisense strands and one sense strand) wherein the at least two sense strands or the at least two antisense strands are separated by a nick or a gap of at least one nucleotide.

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PEPTIDE-DICER SUBSTRATE RNA CONJUGATES AS DELIVERY VEHICLES FOR SIRNA

BACKGROUND OF THE INVENTION

Technical Field of the Invention

The present disclosure relates, generally, to the treatment of disorders by means of RNA interference (RNAi). More specifically, the present disclosure is directed to the targeted delivery of small inhibitory nucleic acid molecules (siRNA), and variants thereof, that are capable of mediating RNAi against gene expression. siRNA described herein may be conjugated to one or more peptides wherein the conjugated peptide(s) facilitates delivery, increases stability, and/or reduces toxicity of the siRNA.

Description of the Related Art

The delivery of nucleic acids into animals and humans (plants) is an important object of molecular biology and clinical research. Specifically, recent developments in the areas of gene therapy, antisense therapy, and RNA interference (RNAi) therapy have created a need to develop more efficient means for introducing nucleic acids into cells.

To date, the most common methods for artificial delivery of nucleic acid into cells use cationic lipids, electroporation, and viral transduction as well as numerous methods that use mechanical or biochemical membrane disruption and/or penetration (e.g., using detergents, microinjection, or particle guns). These methods suffer from a variety of disadvantages. For example, while cationic lipids are used most often for DNA and small interfering RNA (siRNA) delivery *in vitro*, they are generally highly toxic and therefore inappropriate for *in vivo* applications such as the treatment of disease. With viral transduction, there is a possibility that the replication deficient virus used as a delivery vehicle may revert to wild-type thus becoming pathogenic. Electroporation suffers from poor gene-transfer efficiency and cellular damage and therefore has limited clinical application.

RNA interference (RNAi) is emerging as a promising technology for modifying expression of specific genes in plant and animal cells, and is therefore expected to provide useful therapeutics (tools) to treat a wide range of diseases and disorders amenable to treatment by modification of endogenous gene expression. RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs), usually a dsRNA that is homologous in sequence to a portion of a targeted messenger RNA (mRNA). See Fire, et al., *Nature 391*:806, 1998, and Hamilton, et al., *Science*

286:950-951, 1999. The corresponding process in plants is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes and is commonly shared by diverse flora and phyla (Fire, et al., *Trends Genet. 15*:358, 1999).

Introduction of a suitable dsRNA into cells leads to destruction of endogenous, cognate mRNAs (i.e., mRNAs that share substantial sequence identity with the introduced dsRNA). The mechanism by which dsRNA duplexes mediated targeted gene-silencing is thought to be a two step process. First, dsRNAs are fragmented (degraded) by a ribonuclease III enzyme -- referred to as dicer -- into small interfering RNAs (siRNAs) (Hamilton, et al., *supra*; Berstein, et al., *Nature 409*:363, 2001). Short interfering RNAs derived from dicer activity are typically about 21 to about 23 nucleotides in length with two-base 3' overhangs (Kim, et al., *Nature Biotech. 23*(2):222, 2005). Recent evidence suggests that longer dsRNAs (25-30 nucleotides in length) can have greater gene silencing activity than their shorter counterparts (21-mer) directed to the same target site (Kim, et al., *Nature Biotech. 23*(2):222, 2005). Furthermore, certain target sites that are refractory to siRNA mediated gene silencing by 21-mer RNAs can effectively be silenced by longer 27-mer siRNA duplexes. The increased potency of these longer siRNA duplexes is attributed to the fact that they are substrates of the Dicer endonuclease (Kim, et al., *Nature Biotech. 23*(2):222, 2005). Thus, in the context of therapeutic agents, these longer dsRNAs act as precursors which, when processed by dicer, enter the RISC complex and function as mediators of targeted gene silencing.

The second step involves incorporating the siRNA into a multicomponent nuclease complex known as the RNA-induced silencing complex or "RISC." The RISC identifies mRNA substrates through their complementarity to the anti-sense strand of the siRNA duplex, and effectuates silencing of gene expression by binding to and initiating destruction (cleavage) of the targeted mRNA. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex (Elbashir, et al., *Genes Dev. 15*:188, 2001).

There remains a long-standing need in the art for better tools and methods to deliver nucleic acids, peptides and other pharmacological agents into cells, particularly in view of the fact that existing cell delivery techniques are limited by poor efficiency and/or high toxicity of the delivery reagents. Related needs exist for improved methods and formulations to deliver an effective amount, in an active and enduring state, and using non-toxic delivery vehicles to

selected cells, tissues, or compartments to mediate regulation of gene expression in a manner that will alter a phenotype or disease state of the targeted cells.

SUMMARY OF THE INVENTION

The present disclosure addresses these and other related needs by providing, *inter alia*, polynucleotide delivery-enhancing polypeptides/dicer substrate dsRNA conjugates as therapeutic pro-drug delivery systems for the treatment of disease. Upon delivery by the polypeptide into cells, the precursor siRNA is cleaved by dicer, freeing it from the delivery peptide, which then allows the siRNA to enter the RISC complex and target specific genes for post-transcriptional gene silencing. The peptide-dsRNA conjugates described herein provide a promising new approach for improving the delivery of dsRNA therapeutic precursors into cells for the treatment of a wide range of diseases and disorders amenable to treatment by modification of endogenous gene expression. Polynucleotide delivery-enhancing polypeptides/dicer substrate dsRNA conjugates disclosed herein advantageously enhance the protection and/or prophylactic capability of the dsRNA by extending its *in vivo* lifetime.

In one aspect, the present disclosure provides compositions, including pharmaceutical compositions, suitable for administration of one or more double-stranded ribonucleic acid (dsRNA) molecule to an animal, wherein the compositions comprise one or more dsRNA molecule and one or more peptide, wherein each dsRNA molecule comprises about 25 to about 30 base pairs, wherein each peptide comprises between about 5 to about 40 amino acids and comprises an amino acid sequence KVLKQ (SEQ ID NO: 51), and wherein said dsRNA molecule is conjugated to said peptide. Within certain embodiments, the amino acid sequence of the peptide may be selected from the group consisting of: KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 33); KKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 42); VTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 43); AQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 44); KDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 45); KKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 46); KRSRKESYSVYVYKVLKQ (SEQ ID NO: 47); RKESYSVYVYKVLKQ (SEQ ID NO: 41); SYSVYVYKVLKQ (SEQ ID NO: 48); VYVYKVLKQ (SEQ ID NO: 49); YKVLKQ (SEQ ID NO: 50); and KVLKQ (SEQ ID NO: 51).

In other embodiments, the dsRNA has a 5' overhang of 2 or more bp, or a 3' overhang of 2 or more bp wherein the overhang may be on either or both of the sense strand and/or the

antisense strand. In still further embodiments, the dsRNA has no overhang. In yet further embodiments, the dsRNA has strands with lengths of from about 25 bp to about 29 bp. In still further embodiments, the dsRNA molecule contains a sense RNA strand and an antisense RNA strand and a peptide is conjugated to the 5' end of the antisense strand.

In other aspects, the present disclosure provides compositions, including pharmaceutical compositions, suitable for administration of one or more siRNA molecule to an animal, wherein the compositions comprise one or more siRNA molecule and one or more peptide, wherein each siRNA molecule comprises a double-stranded ribonucleotide of between about 25 and about 30 base pairs, and wherein each peptide is between about 5 to about 40 amino acids and includes the amino acid sequence KVLKQ (SEQ ID NO: 51) and wherein the peptide is conjugated to the dsRNA. In an embodiment, the peptide is conjugated to a molecule that binds to a cell in the animal. In another embodiment, the siRNA molecule includes a sequence that is homologous to the sequence of a portion of a TNF-alpha gene.

In yet other embodiments, the present disclosure provides siRNA molecules, and polypeptide conjugates of those siRNA, wherein the siRNA comprise three strands, designated herein as A, B1 and B2 (A:B1B2), wherein B1 and B2 are complementary to, and form base pairs (bp) with, non-overlapping regions of A. Thus, for siRNA molecules within these embodiments, the double-stranded region formed by the annealing of B1 and A is distinct from the double-stranded region formed by the annealing of B2 and A. An A:B1 duplex may be separated from an A:B2 duplex by a "gap" resulting from at least one unpaired nucleotide in the A strand that is positioned between the A:B1 duplex and the A:B2 duplex and that is distinct from any one or more unpaired nucleotide at the 3' end of either or both of the A, B1, and/or B2 strand. Alternatively, an A:B1 duplex may be separated from an A:B2 duplex by a "nick" such that there are no unpaired nucleotides in the A strand that are positioned between the A:B1 duplex and the A:B2 duplex such that the only unpaired nucleotide, if any, is at the 3' end of either or both of the A, B1, and/or B2 strand.

Typically, siRNA according to these aspects of the present disclosure comprise, in sum, between about 15 base-pairs and about 40 base-pairs; more typically, between about 18 and about 35 base-pairs; still more typically between about 20 and 30 base-pairs; and most typically either 21, 22, 23, 24, 25, 26, 27, 28, or 29 base-pairs. The siRNA may, optionally, comprise a single-strand 3' overhang of between 1 nucleotide and 5 nucleotides. Most typically, such a single-strand 3' overhang is 1, 2, 3, or 4 nucleotides.

Thus, such three-strand siRNA of the present disclosure comprise either an A sense strand or an A antisense strand wherein the length of the A strand is between about

15 nucleotides and about 50 nucleotides; more typically the length of the A strand is between about 18 nucleotides and about 40 nucleotides; still more typically, the length of the A strand is between about 20 nucleotides and about 32 nucleotides; and most typically the length of the A strand is 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or 31 nucleotides.

Three-strand siRNA of the present disclosure additionally comprise two or more B strands, designated herein, for example, as B1 and B2, wherein each B strand is complementary to a non-overlapping region of a cognate A strand and wherein a first B strand (B1) is separated from a second B strand (B2) by a nick or a one or more nucleotide gap. Depending upon whether the cognate A strand is a sense strand or an antisense strand, each B strand will be either an antisense strand or a sense strand, respectively. Each B strand (B1, B2, etc.) described herein is, independently, between about 1 nucleotide and about 25 nucleotides in length; more typically between about 4 nucleotides and about 20 nucleotides in length; still more typically between about 5 nucleotides and about 16 nucleotides in length; most typically 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 nucleotides in length.

Depending upon the precise application contemplated, a first B strand (B1) may be separated from a second B strand (B2) by a nick or by a gap. In those embodiments wherein B1 and B2 are separated by a gap, the gap is typically between about 1 nucleotide and about 25 nucleotides; more typically the gap is between about 1 nucleotide and about 15 nucleotides; still more typically the gap is between about 1 nucleotide and about 10 nucleotides; most typically the gap is 1, 2, 3, 4, 5, 6, 7, 8, or 9 nucleotide(s). Each B strand may, independently, terminate with a 5' hydroxyl (i.e., 5'-OH) or may terminate with a 5' phosphate (i.e., 5'-PO_4).

Within other aspects of the present disclosure are provided methods that employ one or more gapped or nicked duplex siRNA molecule(s), and compositions comprising one or more gapped or nicked duplex siRNA molecule(s).

Within certain embodiments, methods disclosed herein comprise the steps of (a) selecting a target gene, wherein the target gene is a target viral gene, for siRNA-mediated gene silencing; (b) designing and/or synthesizing a suitable siRNA molecule(s) for siRNA mediated gene silencing of the target viral gene, wherein the siRNA molecule comprises a gapped or nicked duplex and wherein the gap or nick appears in either the sense strand or in the anti-sense strand; and (c) administering the siRNA molecule to a cell expressing the target viral gene, wherein the siRNA is capable of specifically binding to the corresponding target viral mRNA thereby reducing its expression level in the cell.

Within alternative embodiments, methods disclosed herein comprise the steps of (a) selecting a target gene for siRNA-mediated gene silencing, wherein the target gene is an

endogenous gene and, optionally, wherein the endogenous target gene comprises one or more sequence variation(s) from a corresponding wild-type endogenous gene; (b) designing and/or synthesizing a suitable gapped or nicked duplex siRNA molecule(s) for siRNA mediated gene silencing of the endogenous target gene wherein the siRNA molecule comprises a gapped or nicked duplex and wherein the gap or nick appears in either the sense strand or in the anti-sense strand; and (c) administering the siRNA molecule to a cell expressing the endogenous target gene, wherein the siRNA is capable of specifically binding to the corresponding endogenous target mRNA thereby reducing its expression level in the cell.

It will be understood that methods of the present invention do not require *a priori* knowledge of the nucleotide sequence of every possible gene variant(s) targeted by the gapped or nicked duplex siRNA. Initially, the nucleotide sequence of the siRNA may be selected from a conserved region of the target gene.

Compositions and methods disclosed herein are useful in reducing the titer of a wide variety of target viruses including, but not limited to, retroviruses, such as human immunodeficiency virus (HIV), as well as respiratory viruses, such as human respiratory syncytial virus, human metapneumovirus, human parainfluenza virus 1, human parainfluenza virus 2, human parainfluenza virus 3, human parainfluenza virus 4a, human parainfluenza virus 4b, influenza A virus, influenza B virus, rhinovirus and influenza C virus.

Within another aspect of the present disclosure is provided a method for inhibiting expression of a gene in an animal comprising administering compositions, including pharmaceutical compositions, of one or more double stranded ribonucleic acid (dsRNA) molecules to the animal, wherein the pharmaceutical compositions comprises the dsRNA molecule and a peptide, wherein each dsRNA molecule is between about 25 and about 30 base pairs, wherein each peptide is between about 5 and about 40 amino acids and, typically, includes the amino acid sequence KVLKQ (SEQ ID NO: 51), and wherein each dsRNA molecule is conjugated to a peptide.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE IDENTIFIERS

FIGURE 1: Gel electrophoresis of products of dicer digestion *in vitro* for conjugates dsCoP277nfR952 and dsCoP277nfR950 having polypeptide PN277 conjugated to siRNA N163.

FIGURE 2: RP-HPLC analysis of dicer endonuclease processing kinetics for non-conjugated siRNA N163 duplex. (A) unprocessed N163 duplex, (B) incubation with dicer endonuclease for 1 hr, (C) incubation with dicer endonuclease for 2.5 hr, (D) incubation with dicer endonuclease for 5 hr, and (E) incubation with dicer endonuclease for 7 hr.

FIGURE 3: Chart of RP-HPLC analysis of dicer endonuclease processing kinetics for non-conjugated siRNA N163 duplex shown in FIGURE 2.

FIGURE 4: ESI-MS analysis of 7 hr dicer digestion of non-conjugated N163.

FIGURE 5: ESI-MS analysis of dicer endonuclease processing for a conjugated siRNA having polypeptide PN857 conjugated to siRNA N163. (A) 8 hr control without dicer endonuclease present and (B) 8 hr dicer endonuclease digestion of conjugate.

SEQ ID NO: 1 is the amino acid sequence KRRQRRR of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 2 is the amino acid sequence RQIKIWFQNRRMKWKK of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 3 is the amino acid sequence DAATATRGRSAASRPTERPRAPARSASRPRRPVD of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 4 is the amino acid sequence AAVALLPAVLLALLAP of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 5 is the amino acid sequence AAVLLPVLLPVLLAAP of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 6 is the amino acid sequence VTVLALGALAGVGVG of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 7 is the amino acid sequence GALFLGWLGAAGSTMGA of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 8 is the amino acid sequence MGLGLHLLVLAAALQGA of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 9 is the amino acid sequence LGTYTQDFNKFHTFPQTAIGVGAP of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 10 is the amino acid sequence GWTLNSAGYLLKINLKALAALAKKIL of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 11 is the amino acid sequence TPPKKKRKVEDPKKKK of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 12 is the amino acid sequence RRRRRRR of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 13 is the amino acid sequence KLALKLALKALKAALKLA of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 14 is the amino acid sequence GLFGAIAGFIENGWEG of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 15 is the amino acid sequence FFGAVIGTIALGVATA of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 16 is the amino acid sequence FLGFLLGVGSAIASGV of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 17 is the amino acid sequence GVFVLGFLGFLATAGS of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 18 is the amino acid sequence GAAIGLAWIPYFGPAA of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 19 is the amino acid sequence ACTCPYCKDSEGRGSGDPGKKKQHICHIQGCGKVYGKTSHLRAHLRWHTGERPFMC of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 20 is the amino acid sequence ACTCPNCKDGEKRSGEQGKKKHVCHIPDCGKTFRKTSLLRAHVRLHTGERPFVC of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 21 is the amino acid sequence ACTCPNCKEGGGRGTNLGKKKQHICHIPGCGKVYGKTSHLRAHLRWHSGERPFVC of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 22 is the amino acid sequence ACSCPNCREGEGRGSNEPGKKKQHICHIEGCGKVYGKTSHLRAHLRWHTGERPFIC of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 23 is the amino acid sequence RCTCPNCTNEMSGLPPIVGPDERGRKQHICHIPGCERLYGKASHLKTHLRWHTGERPFLC of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 24 is the amino acid sequence TCDCPNCQEAERLGPAGVHLRKKNIHSCHIPGCGKVYGKTSHLKAHLRWHTGERPFVC of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 25 is the amino acid sequence RCTCPNCKAIKHGDRGSQHTHLCSVPGCGKTYKKTSHLRAHLRKHTGDRPFVC of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 26 is the amino acid sequence PQISLKKKIFFFIFSNFRGDGKSRIHICHLCNKTYGKTSHLRAHLRGHAGNKPFAC of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 27 is the amino acid sequence WWETWKPFQCRICMRNFSTRQARRNHRRRHR of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 28 is the amino acid sequence GKINLKALAALAKKIL of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 29 is the amino acid sequence RVIRVWFQNKRCKDKK of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 30 is the amino acid sequence GRKKRRQRRRPPQGRKKRRQRRRPPQGRKKRRQRRRPPQ of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 31 is the amino acid sequence GEQIAQLIAGYIDIILKKKKSK of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 33 is the amino acid sequence KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 35 is the nucleotide sequence 5'-AUGGUGUGGGUGAGGAGCACAUGGGUG–3' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 36 is the nucleotide sequence 5'-CCCAUGUGCUCCUCACCCACACCdAT–3' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 37 is the amino acid sequence KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 41 is the amino acid sequence RKESYSVYVYKVLKQ of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 42 is the amino acid sequence KKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 43 is the amino acid sequence VTKAQKKDGKKRKRSRKESYSVYVYKVLKQ of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 44 is the amino acid sequence AQKKDGKKRKRSRKESYSVYVYKVLKQ of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 45 is the amino acid sequence KDGKKRKRSRKESYSVYVYKVLKQ of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 46 is the amino acid sequence KKRKRSRKESYSVYVYKVLKQ of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 47 is the amino acid sequence KRSRKESYSVYVYKVLKQ of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 48 is the amino acid sequence SYSVYVYKVLKQ of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 49 is the amino acid sequence VYVYKVLKQ of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 50 is the amino acid sequence YKVLKQ of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 51 is the amino acid sequence KVLKQ of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 52 is the amino acid sequence KGSKKAVTKAQKKEGKKRKRSRKESYSVYVYKVLKQ of an exemplary peptide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 53 is the nucleotide sequence 5'–GCCUGUACCUCAUCUACUCUU–3' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 54 is the nucleotide sequence 3'–UUCGGACAUGGAGUAGAUGAG–5' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 55 is the nucleotide sequence 5'-GCCUCUUCUCCUUCCUGAUCGUGdGdC–3' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 56 is the nucleotide sequence 3'-GUCGGAGAAGAGGAAGGACUAGCACCG–5' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 57 is the nucleotide sequence 5'-GCCUGCUGCACUUUGGAGUGAUCdGdG–3' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 58 is the nucleotide sequence 3'-GACGGACGACGUGAAACCUCACUAGCC–5' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 59 is the nucleotide sequence 5'-CCCAUGUGCUCCUCACCCACACCdAT–3' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 60 is the nucleotide sequence 3'-GUGGGUACACGAGGAGUGGGUGUGGUA–5' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 61 is the nucleotide sequence 5'-ACCUCAUCUACUCCCAGGUCCUCdTdT–3' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 62 is the nucleotide sequence 3'-CAUGGAGUAGAUGAGGGUCCAGGAGAA–5' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 63 is the nucleotide sequence 5'-GCCUGUACCUCAUCUACUCCCAGGUCC–3' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 64 is the nucleotide sequence 5'-GGUCCUGGGAGUAGAUGAGGUACAGGCUU–3' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 65 is the nucleotide sequence 5'-AGACAGCGACCAAAAGAAUUCGGdAdU–3' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 66 is the nucleotide sequence 5'-AUCCGAAUUCUUUUGGUCGCUGUCUdTdT–3' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 67 is the nucleotide sequence 5'-AUGAAGAUCUGUUCCACCAUUGAdAdG–3' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 68 is the nucleotide sequence 5'-CUUCAAUGGUGGAACAGAUCUUCAUdTdT–3' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 69 is the nucleotide sequence 5'-GAUCUGUUCCACCAUUGAAGAACdUdC–3' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 70 is the nucleotide sequence 5'-GAGUUCUUCAAUGGUGGAACAGAUCdTdT–3' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 71 is the nucleotide sequence 5'-UUGAGGAGUGCCUGAUUAAUGAUdCdC–3' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 72 is the nucleotide sequence 5'-GGAUCAUUAAUCAGGCACUCCUCAAdTdT–3' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 73 is the nucleotide sequence 5'-GGAUCUUAUUUCUUCGGAGACAAdTdG–3' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 74 is the nucleotide sequence 5'-CAUUGUCUCCGAAGAAAUAAGAUCCdTdT–3' of an exemplary polynucleotide suitable for generating siRNA-peptide conjugates as described herein.

SEQ ID NO: 75 is the nucleotide sequence of an exemplary 25-mer B strand sequence 5'-GGAUCUUAUUUCUUCGGAGACAAUG-3' suitable for generating siRNA-peptide conjugates as described herein in Table 2.

SEQ ID NO: 76 is the nucleotide sequence of an exemplary 27-mer A strand sequence 3'-TTCCUAGAAUAAAGAAGCCUCUGUUAC-5' suitable for generating siRNA-peptide conjugates as described herein in Table 2.

SEQ ID NOs: 77-132 are nucleotide sequences of A, B1, and B2 strands of exemplary polynucleotides for generating gapped siRNA-peptide conjugates as described herein in Table 2.

SEQ ID NOs: 34, 38-40, 133, and 166 are nucleotide sequences of A, B1, and B2 strands of exemplary polynucleotides for generating nicked siRNA-peptide conjugates as described herein.

DETAILED DESCRIPTION OF INVENTION

The present disclosure is predicated on the observation that short interfering nucleic acid (siNA), or a precursor thereof, employing in combination a polynucleotide delivery-enhancing polypeptide, exhibit improved delivery, enhanced stability, and/or reduced toxicity when administered *in vivo* to a mammal, such as a human. Thus, polynucleotide delivery-enhancing polypeptides disclosed herein may be natural polypeptides or artificial polypeptides that are selected, *inter alia*, for their ability to enhance the intracellular delivery or uptake of polynucleotides, including siNAs, and their precursors.

The siNA, and compositions of the invention, may be admixed or complexed with, or conjugated to, one or more polynucleotide delivery-enhancing polypeptides to form a composition that enhances intracellular delivery of the siNA as compared to delivery resulting from contacting the target cells with a naked siNA (i.e., siNA without the delivery-enhancing polypeptide present).

Selection and Design of Polynucleotide Delivery-enhancing Polypeptides

In certain embodiments of the invention, the polynucleotide delivery-enhancing polypeptide is a histone protein, or a polypeptide or peptide fragment, derivative, analog, or conjugate thereof. Within these embodiments, the siNA is admixed, complexed, or conjugated with one or more full length histone protein(s) or polypeptide(s) corresponding at least in part to a partial sequence of a histone protein, for example of one or more of the following histones: histone H1, histone H2A, histone H2B, histone H3 or histone H4, or one or more polypeptide fragments or derivatives thereof comprising at least a partial sequence of a histone protein, typically at least 5-10 or 10-20 contiguous residues of a native histone protein.

In more detailed embodiments, the siRNA/histone mixture, complex or conjugate is substantially free of amphipathic compounds. In other detailed embodiments, the siNA that is admixed, complexed, or conjugated with the histone protein or polypeptide will comprise a double-stranded RNA, for example a double-stranded RNA that has 30 or fewer nucleotides, and is a short interfering RNA (siRNA). In exemplary embodiments, the histone polynucleotide delivery-enhancing polypeptide comprises a fragment of histone H2B, as exemplified by the polynucleotide delivery-enhancing polypeptide designated PN73 described herein below. In yet additional detailed embodiments, the polynucleotide delivery-enhancing polypeptide may be pegylated to improve stability and/or efficacy, particularly in the context of in vivo administration.

Within additional embodiments of the invention, the polynucleotide delivery-enhancing polypeptide is selected or rationally designed to comprise an amphipathic amino acid sequence.

For example, useful polynucleotide delivery-enhancing polypeptides may be selected which comprise a plurality of non-polar or hydrophobic amino acid residues that form a hydrophobic sequence domain or motif, linked to a plurality of charged amino acid residues that form a charged sequence domain or motif, yielding an amphipathic peptide.

In other embodiments, the polynucleotide delivery-enhancing polypeptide is selected to comprise a protein transduction domain or motif, and a fusogenic peptide domain or motif. A protein transduction domain is a peptide sequence that is able to insert into and preferably transit through the membrane of cells. A fusogenic peptide is a peptide that destabilizes a lipid membrane, for example a plasma membrane or membrane surrounding an endosome, which may be enhanced at low pH. Exemplary fusogenic domains or motifs are found in a broad diversity of viral fusion proteins and in other proteins, for example fibroblast growth factor 4 (FGF-4).

To rationally design polynucleotide delivery-enhancing polypeptides of the invention, a protein transduction domain is employed as a motif that will facilitate entry of the nucleic acid into a cell through the plasma membrane. In certain embodiments, the transported nucleic acid will be encapsulated in an endosome. The interior of endosomes has a low pH resulting in the fusogenic peptide motif destabilizing the membrane of the endosome. The destabilization and breakdown of the endosome membrane allows for the release of the siNA into the cytoplasm where the siNA can associate with a RISC complex and be directed to its target mRNA.

Examples of protein transduction domains for optional incorporation into polynucleotide delivery-enhancing polypeptides of the invention include:

1. TAT protein transduction domain (PTD) (SEQ ID NO: 1) KRRQRRR;
2. Penetratin PTD (SEQ ID NO: 2) RQIKIWFQNRRMKWKK;
3. VP22 PTD (SEQ ID NO: 3) DAATATRGRSAASRPTERPRAPARSASRPRRPVD;
4. Kaposi FGF signal sequences (SEQ ID NO: 4) AAVALLPAVLLALLAP, and SEQ ID NO: 5) AAVLLPVLLPVLLAAP;
5. Human β3 integrin signal sequence (SEQ ID NO: 6) VTVLALGALAGVGVG;
6. gp41 fusion sequence (SEQ ID NO: 7) GALFLGWLGAAGSTMGA;
7. *Caiman crocodylus* Ig(v) light chain (SEQ ID NO: 8) MGLGLHLLVLAAALQGA;
8. hCT-derived peptide (SEQ ID NO: 9) LGTYTQDFNKFHTFPQTAIGVGAP;
9. Transportan (SEQ ID NO: 10) GWTLNSAGYLLKINLKALAALAKKIL;
10. Loligomer (SEQ ID NO: 11) TPPKKKRKVEDPKKKK;
11. Arginine peptide (SEQ ID NO: 12) RRRRRRR; and

12. Amphiphilic model peptide (SEQ ID NO: 13) KLALKLALKALKAALKLA.

Examples of viral fusion peptides fusogenic domains for optional incorporation into polynucleotide delivery-enhancing polypeptides of the invention include:

1. Influenza HA2 (SEQ ID NO: 14) GLFGAIAGFIENGWEG;
2. Sendai F1 (SEQ ID NO: 15) FFGAVIGTIALGVATA;
3. Respiratory Syncytial virus F1 (SEQ ID NO: 16) FLGFLLGVGSAIASGV;
4. HIV gp41 (SEQ ID NO: 17) GVFVLGFLGFLATAGS; and
5. Ebola GP2 (SEQ ID NO: 18) GAAIGLAWIPYFGPAA.

Within yet additional embodiments of the invention, polynucleotide delivery-enhancing polypeptides are provided that incorporate a DNA-binding domain or motif which facilitates polypeptide-siNA complex formation and/or enhances delivery of siNAs within the methods and compositions of the invention. Exemplary DNA binding domains in this context include various "zinc finger" domains as described for DNA-binding regulatory proteins and other proteins identified in Table 1, below (see, e.g., Simpson, et al., *J. Biol. Chem. 278*:28011-28018, 2003).

Table 1

Exemplary Zinc Finger Motifs of Different DNA-Binding Proteins

C_2H_2 Zinc finger motif

	665	675	685	695	705	715
Sp1	ACTCPYCKDS	EGRGSG----	DPGKKKQHIC	HIQGCGKVYG	KTSHLRAHLR	WHTGERPFMC
Sp2	ACTCPNCKDG	EKRS------	GEQGKKKHVC	HIPDCGKTFR	KTSLLRAHVR	LHTGERPFVC
Sp3	ACTCPNCKEG	GGRGTN----	-LGKKKQHIC	HIPGCGKVYG	KTSHLRAHLR	WHSGERPFVC
Sp4	ACSCPNCREG	EGRGSN----	EPGKKKQHIC	HIEGCGKVYG	KTSHLRAHLR	WHTGERPFIC
DrosBtd	RCTCPNCTNE	MSGLPPIVGP	DERGRKQHIC	HIPGCERLYG	KASHLKTHLR	WHTGERPFLC
DrosSp	TCDCPNCQEA	ERLGPAGV--	HLRKKNIHSC	HIPGCGKVYG	KTSHLKAHLR	WHTGERPFVC
CeT22C8.5	RCTCPNCKAI	KHG-------	DRGSQHTHLC	SVPGCGKTYK	KTSHLRAHLR	KHTGDRPFVC
Y40B1A.4	PQISLKKKIF	FFIFSNFR--	GDGKSRIHIC	HL--CNKTYG	KTSHLRAHLR	GHAGNKPFAC

Prosite pattern

C-x(2,4)-C-x(12)-H-x(3)-H

*The table demonstrates a conservative zinc fingerer motif for double strand DNA binding which is characterized by the C-x(2,4)-C-x(12)-H-x(3)-H (SEQ ID NO: 32) motif pattern, which itself can be used to select and design additional polynucleotide delivery-enhancing polypeptides according to the invention.

**The sequences shown in Table 1, for Sp1, Sp2, Sp3, Sp4, DrosBtd, DrosSp, CeT22C8.5, and Y4pB1A.4, are herein assigned SEQ ID NOs: 19, 20, 21, 22, 23, 24, 25, and 26, respectively.

Alternative DNA binding domains useful for constructing polynucleotide delivery-enhancing polypeptides of the invention include, for example, portions of the HIV Tat protein sequence (see, Examples, below).

Within exemplary embodiments of the invention described herein below, polynucleotide delivery-enhancing polypeptides may be rationally designed and constructed by combining any of the foregoing structural elements, domains or motifs into a single polypeptide effective to mediate enhanced delivery of siNAs into target cells. For example, a protein transduction domain of the TAT polypeptide was fused to the N-terminal 20 amino acids of the influenza

virus hemagglutinin protein, termed HA2, to yield one exemplary polynucleotide delivery-enhancing polypeptide herein. Various other polynucleotide delivery-enhancing polypeptide constructs are provided in the instant disclosure, evincing that the concepts of the invention are broadly applicable to create and use a diverse assemblage of effective polynucleotide delivery-enhancing polypeptides for enhancing siNA delivery.

Yet additional exemplary polynucleotide delivery-enhancing polypeptides within the invention may be selected from the following peptides: WWETWKPFQCRICMRNFSTRQARRNHRRRHR (SEQ ID NO: 27); GKINLKALAALAKKIL (SEQ ID NO: 28), RVIRVWFQNKRCKDKK (SEQ ID NO: 29), GRKKRRQRRRPPQGRKKRRQRRRPPQGRKKRRQRRRPPQ (SEQ ID NO: 30), GEQIAQLIAGYIDIILKKKKSK (SEQ ID NO: 31), Poly Lys-Trp, 4:1, MW 20,000-50,000; and Poly Orn-Trp, 4:1, MW 20,000-50,000. Additional polynucleotide delivery-enhancing polypeptides that are useful within the compositions and methods herein comprise all or part of the mellitin protein sequence.

Still other exemplary polynucleotide delivery-enhancing polypeptides are identified in the examples below. Any one or combination of these peptides may be selected or combined to yield effective polynucleotide delivery-enhancing polypeptide reagents to induce or facilitate intracellular delivery of siNAs within the methods and compositions of the invention.

Compositions Comprising a Polynucleotide Delivery-Enhancing Polypeptide, an siRNA, and a Lipid

In more detailed aspects, mixtures, complexes, and/or conjugates comprising one or more siRNA and a polynucleotide delivery-enhancing polypeptide may be optionally combined with (e.g., admixed or complexed with) a cationic lipid, such as LIPOFECTIN®. In this context it is unexpectedly disclosed herein that polynucleotide delivery-enhancing polypeptides complexed or conjugated to a siRNA alone will effectuate delivery of the siNA sufficient to mediate gene silencing by RNAi. It is further disclosed herein that an siRNA/polynucleotide delivery-enhancing polypeptide complex or conjugate will exhibit even greater activity for mediating siNA delivery and gene silencing when admixed or complexed with a cationic lipid, such as lipofectin.

To produce these compositions comprised of a polynucleotide delivery-enhancing polypeptide, siRNA and a cationic lipid, the siRNA and peptide may be mixed together first in a suitable medium such as a cell culture medium, after which the cationic lipid is added to the mixture to form a siRNA/delivery peptide/cationic lipid composition. Optionally, the peptide and cationic lipid can be mixed together first in a suitable medium such as a cell culture medium,

whereafter the siRNA can be added to form the siRNA/delivery peptide/cationic lipid composition.

Examples of useful cationic lipids within these aspects of the invention include N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride, 1,2-bis(oleoyloxy)-3-3-(trimethylammonium)propane, 1,2-dimyristyloxypropyl-3-dimethylhydroxyethylammonium bromide, and dimethyldioctadecylammonium bromide, 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminiu m trifluoracetate, 1,3-dioleoyloxy-2-(6-carboxyspermyl)-propylamid, 5-carboxyspermylglycine dioctadecylamide, tetramethyltetrapalmitoyl spermine, tetramethyltetraoleyl spermine, tetramethyltetralauryl spermine, tetramethyltetramyristyl spermine and tetramethyldioleyl spermine. DOTMA (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl ammonium chloride), DOTAP (1,2-bis(oleoyloxy)-3,3-(trimethylammonium)propane), DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide) or DDAB (dimethyl dioctadecyl ammonium bromide). Polyvalent cationic lipids include lipospermines, specifically DOSPA (2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanamini um trifluoro-acetate) and DOSPER (1,3-dioleoyloxy-2-(6carboxy spermyl)-propyl-amid, and the di- and tetra-alkyl-tetra-methyl spermines, including but not limited to TMTPS (tetramethyltetrapalmitoyl spermine), TMTOS (tetramethyltetraoleyl spermine), TMTLS (tetramethlytetralauryl spermine), TMTMS (tetramethyltetramyristyl spermine) and TMDOS (tetramethyldioleyl spermine) DOGS (dioctadecyl-amidoglycylspermine (TRANSFECTAM®). Other useful cationic lipids are described, for example, in U.S. Patent No. 6,733,777; U.S. Patent No. 6,376,248; U.S. Patent No. 5,736,392; U.S. Patent No. 5,686,958; U.S. Patent No. 5,334,761 and U.S. Patent No. 5,459,127.

Cationic lipids are optionally combined with non-cationic lipids, particularly neutral lipids, for example lipids such as DOPE (dioleoylphosphatidylethanolamine), DPhPE (diphytanoylphosphatidylethanolamine) or cholesterol. A cationic lipid composition composed of a 3:1 (w/w) mixture of DOSPA and DOPE or a 1:1 (w/w) mixture of DOTMA and DOPE (LIPOFECTIN®, Invitrogen) are generally useful in transfecting compositions of this invention. Preferred transfection compositions are those which induce substantial transfection of a higher eukaryotic cell line.

Selection, Design, and Synthesis of Short Interfering Nucleic Acids (siNAs)

In exemplary embodiments, the instant disclosure features compositions comprising a small nucleic acid molecule, such as short interfering nucleic acid (siNA), a short interfering RNA (siRNA), a double-stranded RNA (dsRNA), micro-RNA (mRNA), or a short hairpin RNA

(shRNA), admixed or complexed with, or conjugated to, a polynucleotide delivery-enhancing polypeptide.

As used herein, the term "short interfering nucleic acid", "siNA", "short interfering RNA", "siRNA", "short interfering nucleic acid molecule", "short interfering oligonucleotide molecule", or "chemically-modified short interfering nucleic acid molecule", refers to any nucleic acid molecule capable of inhibiting or down regulating gene expression or viral replication, for example by mediating RNA interference "RNAi" or gene silencing in a sequence-specific manner. Within exemplary embodiments, the siNA is a double-stranded polynucleotide molecule comprising self-complementary sense and antisense regions, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence in a target nucleic acid molecule for down regulating expression, or a portion thereof, and the sense region comprises a nucleotide sequence corresponding to (i.e., which is substantially identical in sequence to) the target nucleic acid sequence or portion thereof.

"siNA" means a small interfering nucleic acid, for example a siRNA, that is a short-length double-stranded nucleic acid (or optionally a longer precursor thereof), and which is not unacceptably toxic in target cells. The length of useful siNAs within the invention will in certain embodiments be optimized at a length of approximately 21 to 23 bp long. However, there is no particular limitation in the length of useful siNAs, including siRNAs. For example, siNAs can initially be presented to cells in a precursor form that is substantially different than a final or processed form of the siNA that will exist and exert gene silencing activity upon delivery, or after delivery, to the target cell. Precursor forms of siNAs may, for example, include precursor sequence elements that are processed, degraded, altered, or cleaved at or following the time of delivery to yield a siNA that is active within the cell to mediate gene silencing. Thus, in certain embodiments, useful siNAs within the invention will have a precursor length, for example, of approximately 100-200 base pairs, 50-100 base pairs, or less than about 50 base pairs, which will yield an active, processed siNA within the target cell. In other embodiments, a useful siNA or siNA precursor will be approximately10 to 49 bp, 15 to 35 bp, or about 21 to 30 bp in length.

Exemplary siNA molecules of the instant disclosure are chemically synthesized. Oligonucleotides are synthesized using protocols known in the art, for example as described in Caruthers, et al., *Methods in Enzymology 211*:3-19, 1992; Thompson, et al., International PCT Publication No. WO 99/54459; Wincott, et al., *Nucleic Acids Res. 23*:2677-2684, 1995; Wincott, et al., *Methods Mol. Bio. 74*:59, 1997; Brennan, et al., *Biotechnol. Bioeng. 61*:33-45, 1998; and Brennan, U.S. Patent No. 6,001,311. The synthesis of a siNA molecule of the invention, which

can be chemically-modified, comprises: (a) synthesis of two complementary strands of the siNA molecule; (b) annealing the two complementary strands together under conditions suitable to obtain a double-stranded siNA molecule. In another embodiment, synthesis of the two complementary strands of the siNA molecule is by solid phase oligonucleotide synthesis. In yet another embodiment, synthesis of the two complementary strands of the siNA molecule is by solid phase tandem oligonucleotide synthesis.

Oligonucleotides (e.g., certain modified oligonucleotides or portions of oligonucleotides lacking ribonucleotides) are synthesized using protocols known in the art, for example as described in Caruthers, et al., *Methods in Enzymology 211*:3-19, 1992; Thompson, et al., International PCT Publication No. WO 99/54459; Wincott, et al., *Nucleic Acids Res. 23*:2677-2684, 1995; Wincott, et al., *Methods Mol. Bio. 74*:59, 1997; Brennan, et al., *Biotechnol Bioeng. 61*:33-45, 1998; and Brennan, U.S. Patent No. 6,001,311. Synthesis of RNA, including certain siNA molecules of the invention, follows general procedures as described, for example, in Usman, et al., *J. Am. Chem. Soc. 109*:7845, 1987; Scaringe, et al., *Nucleic Acids Res. 18*:5433, 1990; and Wincott, et al., *Nucleic Acids Res. 23*:2677-2684, 1995; Wincott, et al., *Methods Mol. Bio. 74*:59, 1997.

In certain embodiments of the invention, as noted above, polynucleotide delivery-enhancing polypeptides are used to facilitate delivery of larger nucleic acid molecules than conventional siNAs, including large nucleic acid precursors of siNAs. For example, the methods and compositions herein may be employed for enhancing delivery of larger nucleic acids that represent "precursors" to desired siNAs, wherein the precursor amino acids may be cleaved or otherwise processed before, during or after delivery to a target cell to form an active siNA for modulating gene expression within the target cell. For example, a siNA precursor polynucleotide may be selected as a circular, single-stranded polynucleotide, having two or more loop structures and a stem comprising self-complementary sense and antisense regions, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence in a target nucleic acid molecule or a portion thereof, and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof, and wherein the circular polynucleotide can be processed either in vivo or in vitro to generate an active siNA molecule capable of mediating RNAi.

In mammalian cells, dsRNAs longer than 30 base pairs can activate the dsRNA-dependent kinase PKR and 2'-5'-oligoadenylate synthetase, normally induced by interferon. The activated PKR inhibits general translation by phosphorylation of the translation factor eukaryotic initiation factor 2α (eIF2α), while 2'-5'-oligoadenylate synthetase causes

nonspecific mRNA degradation via activation of RNase L. By virtue of their small size (referring particularly to non-precursor forms), usually less than 30 base pairs, and most commonly between about 17-19, 19-21, or 21-23 base pairs, the siNAs of the present invention avoid activation of the interferon response.

In contrast to the nonspecific effect of long dsRNA, siRNA can mediate selective gene silencing in the mammalian system. Hairpin RNAs, with a short loop and 19 to 27 base pairs in the stem, also selectively silence expression of genes that are homologous to the sequence in the double-stranded stem. Mammalian cells can convert short hairpin RNA into siRNA to mediate selective gene silencing.

RISC mediates cleavage of single stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex. Studies have shown that 21 nucleotide siRNA duplexes are most active when containing a two nucleotide 3'-overhang. Furthermore, complete substitution of one or both siRNA strands with 2'-deoxy (2'-H) or 2'-O-methyl nucleotides substantially decreases RNAi activity, whereas substitution of the 3'-terminal siRNA overhang nucleotides with deoxy nucleotides (2'-H) has been reported to be tolerated.

Studies have shown that replacing the 3'-overhanging segments of a 21-mer siRNA duplex having 2 nucleotide 3' overhangs with deoxyribonucleotides does not have an adverse effect on RNAi activity. Replacing up to 4 nucleotides on each end of the siRNA with deoxyribonucleotides has been reported to be well tolerated whereas complete substitution with deoxyribonucleotides results in no RNAi activity.

In some embodiments of this invention, the dsRNA has a 5' overhang of 2 or more bp, or a 3' overhang of 2 or more bp, where the overhang may be on either the sense or antisense strand. In some embodiments, the dsRNA has no overhang. In some embodiments, the dsRNA has a length of 27 bp to 29 bp. In some embodiments, the dsRNA molecule contains a sense RNA strand and an antisense RNA strand, and a peptide is conjugated to the 5' end of the antisense strand.

Alternatively, the siNAs can be delivered as single or multiple transcription products expressed by a polynucleotide vector encoding the single or multiple siNAs and directing their expression within target cells. In these embodiments the double-stranded portion of a final transcription product of the siRNAs to be expressed within the target cell can be, for example, 15 to 49 bp, 15 to 35 bp, or about 21 to 30 bp long. Within exemplary embodiments, double-stranded portions of siNAs, in which two strands pair up, are not limited to completely paired nucleotide segments, and may contain nonpairing portions due to mismatch (the

corresponding nucleotides are not complementary), bulge (lacking in the corresponding complementary nucleotide on one strand), overhang, and the like. Nonpairing portions can be contained to the extent that they do not interfere with siNA formation. In more detailed embodiments, a "bulge" may comprise 1 to 2 nonpairing nucleotides, and the double-stranded region of siNAs in which two strands pair up may contain from about 1 to 7, or about 1 to 5 bulges. In addition, "mismatch" portions contained in the double-stranded region of siNAs may be present in numbers from about 1 to 7, or about 1 to 5. Most often in the case of mismatches, one of the nucleotides is guanine, and the other is uracil. Such mismatching may be attributable, for example, to a mutation from C to T, G to A, or mixtures thereof, in a corresponding DNA coding for sense RNA, but other cause are also contemplated. Furthermore, in the present invention the double-stranded region of siNAs in which two strands pair up may contain both bulge and mismatched portions in the approximate numerical ranges specified.

The terminal structure of siNAs of the invention may be either blunt or cohesive (overhanging) as long as the siNA retains its activity to silence expression of target genes. The cohesive (overhanging) end structure is not limited only to the 3' overhang as reported by others. On the contrary, the 5' overhanging structure may be included as long as it is capable of inducing a gene silencing effect such as by RNAi. In addition, the number of overhanging nucleotides is not limited to reported limits of 2 or 3 nucleotides, but can be any number as long as the overhang does not impair gene silencing activity of the siNA. For example, overhangs may comprise from about 1 to 8 nucleotides, more often from about 2 to 4 nucleotides.

The length of siNAs having cohesive (overhanging) end structure may be expressed in terms of the paired duplex portion and any overhanging portion at each end. For example, a 25/27-mer siNA duplex with a 2-bp 3' antisense overhang has a 25-mer sense strand and a 27-mer antisense strand, where the paired portion has a length of 25 bp.

Furthermore, since the overhanging sequence may have low specificity to a target gene, it is not necessarily complementary (antisense) or identical (sense) to the target gene sequence. Furthermore, as long as the siNA is able to maintain its gene silencing effect on the target gene, it may contain a low molecular weight structure (for example a natural RNA molecule such as tRNA, rRNA or viral RNA, or an artificial RNA molecule), for example, in the overhanging portion at one end.

In addition, the terminal structure of the siNAs may have a stem-loop structure in which ends of one side of the double-stranded nucleic acid are connected by a linker nucleic acid, e.g., a linker RNA. The length of the double-stranded region (stem-loop portion) can be, for example, 15 to 49 bp, often 15 to 35 bp, and more commonly about 21 to 30 bp long. Alternatively, the

length of the double-stranded region that is a final transcription product of siNAs to be expressed in a target cell may be, for example, approximately 15 to 49 bp, 15 to 35 bp, or about 21 to 30 bp long. When linker segments are employed, there is no particular limitation in the length of the linker as long as it does not hinder pairing of the stem portion. For example, for stable pairing of the stem portion and suppression of recombination between DNAs coding for this portion, the linker portion may have a clover-leaf tRNA structure. Even if the linker has a length that would hinder pairing of the stem portion, it is possible, for example, to construct the linker portion to include introns so that the introns are excised during processing of a precursor RNA into mature RNA, thereby allowing pairing of the stem portion. In the case of a stem-loop siRNA, either end (head or tail) of RNA with no loop structure may have a low molecular weight RNA. As described above, these low molecular weight RNAs may include a natural RNA molecule, such as tRNA, rRNA or viral RNA, or an artificial RNA molecule.

The siNA can also comprise a single stranded polynucleotide having nucleotide sequence complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof (for example, where such siNA molecule does not require the presence within the siNA molecule of nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof), wherein the single stranded polynucleotide can further comprise a terminal phosphate group, such as a 5'-phosphate (see for example, Martinez, et al., *Cell. 110*:563-574, 2002, and Schwarz, et al., *Molecular Cell 10*:537-568, 2002), or 5',3'-diphosphate.

As used herein, the term siNA molecule is not limited to molecules containing only naturally-occurring RNA or DNA, but also encompasses chemically-modified nucleotides and non-nucleotides.

In certain embodiments, the short interfering nucleic acid molecules of the invention lack 2'-hydroxy (2'-OH) containing nucleotides. In certain embodiments short interfering nucleic acids do not require the presence of nucleotides having a 2'-hydroxy group for mediating RNAi and as such, short interfering nucleic acid molecules of the invention optionally do not include any ribonucleotides (e.g., nucleotides having a 2'-OH group). Such siNA molecules that do not require the presence of ribonucleotides within the siNA molecule to support RNAi can however have an attached linker or linkers or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. Optionally, siNA molecules can comprise ribonucleotides at about 5, 10, 20, 30, 40, or 50% of the nucleotide positions.

As used herein, the term siNA is meant to be equivalent to other terms used to describe nucleic acid molecules that are capable of mediating sequence specific RNAi, for example short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (mRNA), short hairpin

RNA (shRNA), short interfering oligonucleotide, short interfering nucleic acid, short interfering modified oligonucleotide, chemically-modified siRNA, post-transcriptional gene silencing RNA (ptgsRNA), and others.

In other embodiments, siNA molecules for use within the invention may comprise separate sense and antisense sequences or regions, wherein the sense and antisense regions are covalently linked by nucleotide or non-nucleotide linker molecules, or are alternately non-covalently linked by ionic interactions, hydrogen bonding, van der waals interactions, hydrophobic interactions, and/or stacking interactions.

"Antisense RNA" is an RNA strand having a sequence complementary to a target gene mRNA, and thought to induce RNAi by binding to the target gene mRNA. "Sense RNA" has a sequence complementary to the antisense RNA, and annealed to its complementary antisense RNA to form siRNA. These antisense and sense RNAs have been conventionally synthesized with an RNA synthesizer.

As used herein, the term "RNAi construct" is a generic term used throughout the specification to include small interfering RNAs (siRNAs), hairpin RNAs, and other RNA species which can be cleaved *in vivo* to form siRNAs. RNAi constructs herein also include expression vectors (also referred to as RNAi expression vectors) capable of giving rise to transcripts which form dsRNAs or hairpin RNAs in cells, and/or transcripts which can produce siRNAs *in vivo*. Optionally, the siRNA include single strands or double strands of siRNA.

An siHybrid molecule is a double-stranded nucleic acid that has a similar function to siRNA. Instead of a double-stranded RNA molecule, an siHybrid is comprised of an RNA strand and a DNA strand. Preferably, the RNA strand is the antisense strand as that is the strand that binds to the target mRNA. The siHybrid created by the hybridization of the DNA and RNA strands have a hybridized complementary portion and preferably at least one 3' overhanging end.

siNAs for use within the invention can be assembled from two separate oligonucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary (i.e., each strand comprises nucleotide sequence that is complementary to nucleotide sequence in the other strand; such as where the antisense strand and sense strand form a duplex or double stranded structure, for example wherein the double stranded region is about 19 base pairs). The antisense strand may comprise a nucleotide sequence that is complementary to a nucleotide sequence in a target nucleic acid molecule or a portion thereof, and the sense strand may comprise a nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. Alternatively, the siNA can be assembled from

a single oligonucleotide, where the self-complementary sense and antisense regions of the siNA are linked by means of a nucleic acid-based or non-nucleic acid-based linker(s).

Within additional embodiments, siNAs for intracellular delivery according to the methods and compositions of the invention can be a polynucleotide with a duplex, asymmetric duplex, hairpin or asymmetric hairpin secondary structure, having self-complementary sense and antisense regions, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence in a separate target nucleic acid molecule or a portion thereof, and the sense region comprises a nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof.

Within still further embodiments, the present disclosure provides three-stranded siNA, and compositions thereof, that are admixed or complexed with, or conjugated to, one or more polynucleotide delivery-enhancing polypeptide. siRNA molecules described herein comprise three strands, A, B1 and B2 (A:B1B2), wherein B1 and B2 strands are complementary to, and base pair (bp) with, non-overlapping regions of a cognate A strand and wherein a B1 strand is separated from a corresponding B2 strand by a nick or by a gap. The siRNA molecules described herein are able to initiate an RNA interference cascade resulting in the modification of the expression of a target messenger RNA (mRNA).

siRNA molecules described herein comprise three or more strands such as, for example, A, B1, and B2 (A:B1B2) wherein B1 and B2 are complementary to, and form base pairs (bp) with, non-overlapping regions of A; wherein the double-stranded region formed by the annealing of B1 and A is distinct from, and non-overlapping with, the double-stranded region formed by the annealing of B2 and A; and wherein the A:B1 duplex is separated from the A:B2 duplex by a "gap" resulting from at least one unpaired nucleotide in the A strand that is positioned between the A:B1 duplex and the A:B2 duplex and that is distinct from any one or more unpaired nucleotide at the 3' end of either or both of the A, B1, and/or B2 strand.

Alternatively, siRNA molecules described in this disclosure comprise three or more strands such as, for example, A, B1, and B2 (A:B1B2) wherein B1 and B2 are complementary to, and form base pairs (bp) with, non-overlapping regions of A; wherein the double-stranded region formed by the annealing of B1 and A is distinct from, and non-overlapping with, the double-stranded region formed by the annealing of B2 and A; and wherein the A:B1 duplex is separated from the A:B2 duplex by a "nick" between the A:B1 duplex and the A:B2 duplex.

In one embodiment, A:B1B2 includes, in sum, between about 14 base pairs and about 40 base pairs. In this embodiment, A represents the sense strand and B1B2 represents the antisense strand. A is 15-50 nucleotides in length, and B1 and B2 are each, individually,

1-20 nucleotides; and the combined length of B1+B2 is between about 8 nucleotides and about 40 nucleotides.

In another embodiment, A:B1B2 includes, in sum, between about 16 base pairs and about 40 base pairs. In this embodiment, A represents the sense strand and B1B2 represents the antisense strand. A is 20-40 nucleotides in length, and B1 and B2 are each, individually, 1-15 nucleotides; and the combined length of B1+B2 is between about 10 nucleotides and about 30 nucleotides.

In another embodiment, A:B1B2 includes, in sum, between about 14 base pairs and about 40 base pairs. In this embodiment, A represents the antisense strand and B1B2 represents the sense strand. A is 15-50 nucleotides in length, and B1 and B2 are each, individually, 1-20 nucleotides; and the combined length of B1+B2 is between about 8 nucleotides and about 40 nucleotides.

In another embodiment, A:B1B2 includes, in sum, between about 16 base pairs and about 40 base pairs. In this embodiment, A represents the antisense strand and B1B2 represents the sense strand. A is 20-40 nucleotides, and B1 and B2 are each, individually, 1-15 nucleotides; and the combined length of B1+B2 is between about 10 nucleotides and about 30 nucleotides.

As indicated above, three-stranded siRNA disclosed herein typically comprise, in sum, between about 15 base-pairs and about 40 base-pairs; more typically, between about 18 and about 35 base-pairs; still more typically between about 20 and 30 base-pairs; and most typically either 21, 22, 23, 24, 25, 26, 27, 28, or 29 base-pairs. Within certain embodiments, the siRNA may, optionally, comprise a single-strand 3' overhang of between 1 nucleotide and 5 nucleotides. Most typically, such a single-strand 3' overhang is 1, 2, 3, or 4 nucleotides.

Such siRNA comprise either an A sense strand or an A antisense strand wherein the length of the A strand is between about 15 nucleotides and about 50 nucleotides; more typically the length of the A strand is between about 18 nucleotides and about 40 nucleotides; still more typically, the length of the A strand is between about 20 nucleotides and about 32 nucleotides; and most typically the length of the A strand is 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or 31 nucleotides.

siRNA of the present disclosure additionally comprise two or more B strands, designated herein, for example, as B1 and B2, wherein each B strand is complementary to a non-overlapping region of a cognate A strand and wherein a first B strand (B1) is separated from a second B strand (B2) by a nick or a one or more nucleotide gap. Depending upon whether the cognate A strand is a sense strand or an antisense strand, each B strands will be either an antisense strand or a sense strand, respectively. Each B strand (B1, B2, etc.) described herein is,

independently, between about 1 nucleotide and about 25 nucleotides in length; more typically between about 4 nucleotides and about 20 nucleotides in length; still more typically between about 5 nucleotides and about 16 nucleotides in length; most typically 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 nucleotides in length.

Once synthesized A, B1 and B2 strands are annealed to form the siRNA molecules. The annealing reaction is determined by complementarity. A first B strand (B1) may be separated from a second B strand (B2) by a nick or by a gap. Thus, nucleotides on the A strand for which no cognate nucleotide are present on a B strand remain unpaired and appear as a gap in the siRNA molecule. The size of the gap will depend on the number of unpaired nucleotides in the A strand that are positioned between the B1 and B2 strands. A nick is formed if an A strand base pairs with a B1 strand and a B2 strand such that no nucleotides on the A strand remain unpaired in the region between the B1 strand and the B2 strand.

In those embodiments in which B1 and B2 are separated by a gap, the gap is typically between about 1 nucleotide and about 25 nucleotides; more typically the gap is between about 1 nucleotide and about 15 nucleotides; still more typically the gap is between about 1 nucleotide and about 10 nucleotides; most typically the gap is 1, 2, 3, 4, 5, 6, 7, 8, or 9 nucleotide(s).

Each B strand may, independently, terminate with a 5' hydroxyl (i.e., 5'-OH) or may terminate with a 5' phosphate (i.e., 5'-PO_4). Typically, synthetic RNA molecules contain terminal 3' and 5' hydroxyls. Thus the 3'-OH of a first B strand may be juxtaposed immediately adjacent to a 5'-OH of a second B strand. In certain embodiments, it may be desirable to use a second B strand wherein the 5' terminus contains a 5'-PO_4 such that the 3'-OH of a first B strand is juxtaposed immediately adjacent to a 5'-PO_4 of a second B strand. In such instances, the addition of a phosphate to the 5' ends of a B strand may be achieved by adding a PO_4 group to a single-stranded (ss) RNA molecule by exploiting the catalytic activity of RNA kinase by methodology that are well known and readily available in the art. After the kinase reaction is performed, the A, B1, and B2 strands are annealed to each other such that the B strands are oriented 5' B1 pB2 3'.

The following siRNA molecules represent specific non-limiting exemplary embodiments of the gapped or nicked siRNA molecules that may be suitably conjugated to one or more polypeptide as described herein above.

i. <u>Gapped duplex siRNA molecules</u>

Certain exemplary gapped duplex siRNA molecules described herein are based upon a 25-mer sense B strand sequence 5'- GGAUCUUAUUUCUUCGGAGACAAUG-3' (SEQ ID NO: 75) and a 27-mer antisense A strand sequence

3'-TTCCUAGAAUAAAGAAGCCUCUGUUAC-5' (SEQ ID NO: 76). Alternative exemplary gapped duplex siRNA molecules are based upon a 25-mer antisense B strand sequence 5'-GGAUCUUAUUUCUUCGGAGACAAUG-3' (SEQ ID NO: 75) and a 27-mer sense A strand sequence 3'-TTCCUAGAAUAAAGAAGCCUCUGUUAC-5' (SEQ ID NO: 76).

Such exemplary siRNA molecules comprise three strands A, B1, and B2 (A:B1B2) wherein B1 and B2 are complementary to, and form base pairs (bp) with, non-overlapping regions of A; wherein the double-stranded region formed by the annealing of B1 and A is distinct from the double-stranded region formed by the annealing of B2 and A; and wherein the A:B1 duplex is separated from the A:B2 duplex by a "gap" resulting from one or more unpaired nucleotide in the A strand that is between the A:B1 duplex and the A:B2 duplex and that is distinct from any one or more unpaired nucleotide at the 3' end of either or both of the A, B1, and/or B2 strand.

Within certain aspects of these embodiments, A:B1B2 consists of between about 14 and about 24 total base pairs; wherein an A strand (sense) is between about 19 nucleotides and about 27 nucleotides; and a B1 (antisense) strand and a B2 (antisense) strand that are each, individually, between about 1 nucleotide and about 18 nucleotides; and wherein the combined length of B1+B2 is between about 13 nucleotides and about 23 nucleotides.

Within other aspects of these embodiments, A:B1B2 consists of between about 16 and about 22 total base pairs, wherein an A strand (sense) is between about 19 nucleotides and about 23 nucleotides; and a B1 (antisense) strand and a B2 (antisense) strand that are each, individually, between about 1 nucleotide and about 15 nucleotides; and wherein the combined length of B1+B2 is between about 13 nucleotides and about 23 nucleotides.

Within further aspects of these embodiments, A:B1B2 consists of between about 14 and about 24 total base pairs, wherein an A strand (antisense) is between about 14 nucleotides and about 27 nucleotides; and a B1 (sense) strand and a B2 (sense) strand that are each, individually, between about 1 nucleotide and about 18 nucleotides; and wherein the combined length of B1+B2 is between about 18 nucleotides and about 24 nucleotides.

Within yet other aspects of these embodiments, A:B1B2 consists of between about 14 base pairs and about 22 total base pairs; wherein an A strand (antisense) is between about 16 nucleotides and about 22 nucleotides; and a B1 (sense) strand and a B2 (sense) strand that are each, individually, between about 1 nucleotide and about 15 nucleotides; and wherein the combined length of B1+B2 is between about 18 nucleotides and about 22 nucleotides.

Representative siRNA according to these embodiments are presented in Table 2.

Table 2

Representative siRNA

A:B1B2	Sequence Identifier	Sequence
6-nuc B1 (sense) 18-nuc B2 (sense) 27-nuc A (antisense) 1-nuc gap	SEQ ID NO: 77 SEQ ID NO: 78 SEQ ID NO: 76	5'GGAUCU3' 5'AUUUCUUCGGAGACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
8-nuc B1 (sense) 16-nuc B2 (sense) 27-nuc A (antisense) 1-nuc gap	SEQ ID NO: 79 SEQ ID NO: 80 SEQ ID NO: 76	5'GGAUCUUA3' 5'UUCUUCGGAGACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
10-nuc B1 (sense) 14-nuc B2 (sense) 27-nuc A (antisense) 1-nuc gap	SEQ ID NO: 81 SEQ ID NO: 82 SEQ ID NO: 76	5'GGAUCUUAUU3' 5'CUUCGGAGACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
12-nuc B1 (sense) 12-nuc B2 (sense) 27-nuc A (antisense) 1-nuc gap	SEQ ID NO: 83 SEQ ID NO: 84 SEQ ID NO: 76	5'GGAUCUUAUUUC3' 5'UCGGAGACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
14-nuc B1 (sense) 10-nuc B2 (sense) 27-nuc A (antisense) 1-nuc gap	SEQ ID NO: 85 SEQ ID NO: 86 SEQ ID NO: 76	5'GGAUCUUAUUUCUU3' 5'GGAGACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
16-nuc B1 (sense) 8-nuc B2 (sense) 27-nuc A (antisense) 1-nuc gap	SEQ ID NO: 87 SEQ ID NO: 88 SEQ ID NO: 76	5'GGAUCUUAUUUCUUCG3' 5'AGACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
18-nuc B1 (sense) 6-nuc B2 (sense) 27-nuc A (antisense) 1-nuc gap	SEQ ID NO: 89 SEQ ID NO: 90 SEQ ID NO: 76	5'GGAUCUUAUUUCUUCGGA3' 5'ACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
6-nuc B1 (sense) 17-nuc B2 (sense) 27-nuc A (antisense) 2-nuc gap	SEQ ID NO: 91 SEQ ID NO: 92 SEQ ID NO: 76	5'GGAUCU3' 5'UUUCUUCGGAGACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
8-nuc B1 (sense) 15-nuc B2 (sense) 27-nuc A (antisense) 2-nuc gap	SEQ ID NO: 93 SEQ ID NO: 94 SEQ ID NO: 76	5'GGAUCUUA3' 5'UCUUCGGAGACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
10-nuc B1 (sense) 13-nuc B2 (sense) 27-nuc A (antisense) 2-nuc gap	SEQ ID NO: 95 SEQ ID NO: 96 SEQ ID NO: 76	5'GGAUCUUAUU3' 5'UUCGGAGACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'

A:B1B2	Sequence Identifier	Sequence
12-nuc B1 (sense) 11-nuc B2 (sense) 27-nuc A (antisense) 2-nuc gap	SEQ ID NO: 97 SEQ ID NO: 98 SEQ ID NO: 76	5'GGAUCUUAUUUC3' 5'CGGAGACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
14-nuc B1 (sense) 9-nuc B2 (sense) 27-nuc A (antisense) 2-nuc gap	SEQ ID NO: 99 SEQ ID NO: 100 SEQ ID NO: 76	5'GGAUCUUAUUUCUU3' 5'GAGACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
16-nuc B1 (sense) 7-nuc B2 (sense) 27-nuc A (antisense) 2-nuc gap	SEQ ID NO: 101 SEQ ID NO: 102 SEQ ID NO: 76	5'GGAUCUUAUUUCUUCG3' 5'GACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
18-nuc B1 (sense) 5-nuc B2 (sense) 27-nuc A (antisense) 2-nuc gap	SEQ ID NO: 103 SEQ ID NO: 104 SEQ ID NO: 76	5'GGAUCUUAUUUCUUCGGA3' 5'CAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
6-nuc B1 (sense) 15-nuc B2 (sense) 27-nuc A (antisense) 4-nuc gap	SEQ ID NO: 105 SEQ ID NO: 106 SEQ ID NO: 76	5'GGAUCU3' 5'UCUUCGGAGACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
8-nuc B1 (sense) 13-nuc B2 (sense) 27-nuc A (antisense) 4-nuc gap	SEQ ID NO: 107 SEQ ID NO: 108 SEQ ID NO: 76	5'GGAUCUUA3' 5'UUCGGAGACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
10-nuc B1 (sense) 11-nuc B2 (sense) 27-nuc A (antisense) 4-nuc gap	SEQ ID NO: 109 SEQ ID NO: 110 SEQ ID NO: 76	5'GGAUCUUAUU3' 5'CGGAGACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
12-nuc B1 (sense) 9-nuc B2 (sense) 27-nuc A (antisense) 4-nuc gap	SEQ ID NO: 111 SEQ ID NO: 112 SEQ ID NO: 76	5'GGAUCUUAUUUC3' 5'GAGACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
14-nuc B1 (sense) 7-nuc B2 (sense) 27-nuc A (antisense) 4-nuc gap	SEQ ID NO: 113 SEQ ID NO: 114 SEQ ID NO: 76	5'GGAUCUUAUUUCUU3' 5'GACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
16-nuc B1 (sense) 5-nuc B2 (sense) 27-nuc A (antisense) 4-nuc gap	SEQ ID NO: 115 SEQ ID NO: 116 SEQ ID NO: 76	5'GGAUCUUAUUUCUUCG3' 5'CAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
18-nuc B1 (sense) 3-nuc B2 (sense) 27-nuc A (antisense) 4-nuc gap	SEQ ID NO: 117 SEQ ID NO: 118 SEQ ID NO: 76	5'GGAUCUUAUUUCUUCGGA3' 5'AUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'

A:B1B2	Sequence Identifier	Sequence
6-nuc B1 (sense) 13-nuc B2 (sense) 27-nuc A (antisense) 6-nuc gap	SEQ ID NO: 119 SEQ ID NO: 120 SEQ ID NO: 76	5'GGAUCU3' 5'UUCGGAGACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
8-nuc B1 (sense) 11-nuc B2 (sense) 27-nuc A (antisense) 6-nuc gap	SEQ ID NO: 121 SEQ ID NO: 122 SEQ ID NO: 76	5'GGAUCUUA3' 5'CGGAGACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
10-nuc B1 (sense) 9-nuc B2 (sense) 27-nuc A (antisense) 6-nuc gap	SEQ ID NO: 123 SEQ ID NO: 124 SEQ ID NO: 76	5'GGAUCUUAUU3' 5'GAGACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
12-nuc B1 (sense) 7-nuc B2 (sense) 27-nuc A (antisense) 6-nuc gap	SEQ ID NO: 125 SEQ ID NO: 126 SEQ ID NO: 76	5'GGAUCUUAUUUC3' 5'GACAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
14-nuc B1 (sense) 5-nuc B2 (sense) 27-nuc A (antisense) 6-nuc gap	SEQ ID NO: 127 SEQ ID NO: 128 SEQ ID NO: 76	5'GGAUCUUAUUUCUU3' 5'CAAUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
16-nuc B1 (sense) 3-nuc B2 (sense) 27-nuc A (antisense) 6-nuc gap	SEQ ID NO: 129 SEQ ID NO: 130 SEQ ID NO: 76	5'GGAUCUUAUUUCUUCG3' 5'AUG3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'
18-nuc B1 (sense) 1-nuc B2 (sense) 27-nuc A (antisense) 6-nuc gap	SEQ ID NO: 131 SEQ ID NO: 132 SEQ ID NO: 76	5'GGAUCUUAUUUCUUCGGA3' 5'G3' 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5'

It will be recognized that the precise nucleotide sequences of the A, B1, and B2 strands may vary with the proviso that the siRNA molecule will include two stretches of continuous base pairs each stretch of continuous base pairs being separated by a gap of at least one nucleotide, which corresponds to one or more unpaired base in the single antisense A strand or in the single sense A strand.

ii. Nicked duplex siRNA molecules

A "p" in the sequences set forth below indicates a nick in an RNA strand with the 5' phosphate attached to the B2 strand. A "*" in the sequences set forth below indicates a nick in an RNA strand without the 5' phosphate attached to the B2 strand. The "p" and the "*" also indicate the location of the nick. In all cases, the A strand is left intact (not nicked) -- the gap that appears in the A strand is to preserve the proper sequence alignment (due to sequence homology)

of the siRNA molecules. A "***T***" (bold italicized font) in the sequences set forth below indicates the presence of a ribothymidine (rT) molecule at that position. A "**T**" (bold font) in the sequences set forth below indicates the presence of a deoxyribothymidine (dT) molecule at that position. Continuous strands, i.e., those without gaps or nicks, are underlined.

1. Control dsRNA

SEN GGAUCUUAUUUCUUCGGAG**TT**-3' (SEQ ID NO: 133)
ASN CUCCGAAGAAAUAAGAUCC**TT**-3' (SEQ ID NO: 134)

2. Nicked sense strand without 5' phosphate

B1 B2
SEN GGAUCUUAUUU*CUUCGGAG**TT**-3' (SEQ ID NOs: 135 and 136)
ASN CUCCGAAGAAA UAAGAUCC**TT**-3' (SEQ ID NO: 137)
A

3. Nicked sense strand with 5' phosphate

B1 B2
SEN GGAUCUUAUUUp**C**UUCGGAG**TT**-3' (SEQ ID NOs: 138 and 139)
ASN CUCCGAAGAAA UAAGAUCC**TT**-3' (SEQ ID NO: 140)
A

4. Nicked sense strand with 5' phosphate, both strands fully modified with rT molecules

B1 B2
SEN GGA***T***C***TT***A***TTT***p**C*****TT***CGGAG**TT**-3' (SEQ ID NOs: 141 and 142)
ASN C***T***CCGAAGAAA ***T***AAGA***T***CC**TT**-3' (SEQ ID NO: 143)
A

5. Nicked sense strand without 5' phosphate, both strands fully modified with rT molecules

B1 B2
SEN GGATCTTATTT*CTTCGGAGTT-3' (SEQ ID NOs: 144 and 145)
ASN C***T***CCGAAGAAA ***T***AAGA***T***CC**TT**-3' (SEQ ID NO: 146)
A

6. Nicked antisense strand without 5' phosphate, antisense strand fully modified with rT molecules, sense strand unmodified (control)

```
                     A
SEN   GGATCTTATT TCTTCGGAGTT-3'  (SEQ ID NO: 147)
ASN   CTCCGAAGAA*ATAAGATCCTT-3'  (SEQ ID NOs: 148 and 149)
           B1          B2
```

7. Nicked antisense strand with 5' phosphate, antisense strand fully modified with rT molecules, sense strand unmodified (control)

```
                     A
SEN   GGATCTTATT TCTTCGGAGTT-3'  (SEQ ID NO: 150)
ASN   CTCCGAAGAApATAAGATCCTT-3'  (SEQ ID NOs: 151 and 152)
           B1          B2
```

8. ID: NickedS-WT: Duplex with nicked sense strand

```
           B1          B2
SEN   GGAUCUUAUUU*CUUCGGAGTT-3'  (SEQ ID NOs: 153 and 154)
ASN   CUCCGAAGAAA UAAGAUCCTT-3'  (SEQ ID NO: 155)
                     A
```

9. ID: NickedS-WT: Duplex with nicked sense strand; B2 is 5' phosphorylated

```
           B1          B2
SEN   GGAUCUUAUUUpCUUCGGAGTT-3'  (SEQ ID NOs: 156 and 157)
ASN   CUCCGAAGAAA UAAGAUCCTT-3'  (SEQ ID NO: 158)
                     A
```

10. ID: NickedS-RT: Duplex with nicked sense strand; fully rT modified

```
               B1            B2
SEN   GGATCTTATTT*CTTCGGAGTT-3'  (SEQ ID NOs: 159 and 160)
ASN   CTCCGAAGAAA TAAGATCCTT-3'  (SEQ ID NO: 161)
                     A
```

11. ID: NickedS-RT: Duplex with nicked sense strand; B2 is 5' phosphorylated; fully rT modified

```
               B1            B2
SEN   GGATCTTATTTpCTTCGGAGTT-3'  (SEQ ID NOs: 162 and 163)
ASN   CTCCGAAGAAA TAAGATCCTT-3'  (SEQ ID NO: 164)
                     A
```

12. ID: NickedA-RT: Duplex with nicked antisense strand; fully rT modified

```
                    A
SEN   GGATCTTATT TCTTCGGAGTT-3'  (SEQ ID NO: 165)
ASN   CTCCGAAGAA*ATAAGATCCTT-3'  (SEQ ID NOs: 166 and 34)
               B1             B2
```

13. ID: NickedA-RT: Duplex with nicked antisense strand; B2 is 5' phosphorylated; fully rT modified

```
                    A
SEN   GGATCTTATT TCTTCGGAGTT-3'  (SEQ ID NO: 38)
ASN   CTCCGAAGAApATAAGATCCTT-3'  (SEQ ID NOs: 39 and 40)
               B1             B2
```

iii. <u>Gapped or nicked duplex siRNA molecules closed with linkers</u>

In another embodiment, gapped or nicked duplex siRNA molecules according to the present disclosure may be "closed" with a linker molecule. The resulting molecule should be much more stable, i.e., have a higher T_m and be more resistant to degradation in the host cell.

Examples of such linkers according to the present disclosure include, but are not limited to, abasic linkers, e.g., abasic nucleotides, abasic propanediols, and a host of small C6-type linkers.

A non-nucleotide linker may be comprised of an abasic nucleotide, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, polyhydrocarbon, or other polymeric compounds (e.g., polyethylene glycols such as those having between 2 and 100 ethylene glycol units). Specific examples include those described by Seela and Kaiser, *Nucleic Acids Res. 18*:6353, 1990 and *Nucleic Acids Res. 15*:3113, 1987; Cload and Schepartz, *J. Am. Chem. Soc. 113*:6324, 1991; Richardson and Schepartz, *J. Am. Chem. Soc. 113*:5109, 1991; Ma, et al., *Nucleic Acids Res. 21*:2585, 1993 and *Biochemistry 32*:1751, 1993; Durand, et al., *Nucleic Acids Res. 18*:6353, 1990; McCurdy, et al., *Nucleosides & Nucleotides 10*:287, 1991; Jschke, et al., *Tetrahedron Lett. 34*:301, 1993; Ono, et al., *Biochemistry 30*:9914, 1991; Arnold, et al., International Publication No. WO 89/02439; Usman, et al., International Publication No. WO 95/06731; Dudycz, et al., International Publication No. WO 95/11910 and Ferentz and Verdine, *J. Am. Chem. Soc. 113*:4000, 1991. A "non-nucleotide" further means any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound can be abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymidine, for example at the C1 position of the sugar.

This presence of the linker should not interfere significantly with RISC activity. Preferably, administration of the linker-containing gapped or nicked duplex siRNA molecules of the present disclosure will result in substantially the same level of RNA interference as that seen when employing the corresponding non-linked gapped or nicked duplex siRNA molecules. Most preferably, administration of the linker-containing gapped or nicked duplex siRNA molecules of the present disclosure will result in an elevated level of RNA interference from that seen when employing the corresponding non-linked gapped or nicked duplex siRNA molecules.

Chemical Modification of Small Inhibitory Nucleic Acids (siNA)

In a non-limiting example, the introduction of chemically-modified nucleotides into nucleic acid molecules provides a powerful tool in overcoming potential limitations of in vivo stability and bioavailability inherent to native RNA molecules that are delivered exogenously. For example, the use of chemically-modified nucleic acid molecules can enable a lower dose of a particular nucleic acid molecule for a given therapeutic effect since chemically-modified nucleic acid molecules tend to have a longer half-life in serum. Furthermore, certain chemical

modifications can improve the bioavailability of nucleic acid molecules by targeting particular cells or tissues and/or improving cellular uptake of the nucleic acid molecule. Therefore, even if the activity of a chemically-modified nucleic acid molecule is reduced as compared to a native nucleic acid molecule, for example, when compared to an all-RNA nucleic acid molecule, the overall activity of the modified nucleic acid molecule can be greater than that of the native molecule due to improved stability and/or delivery of the molecule. Unlike native unmodified siNA, chemically-modified siNA can also minimize the possibility of activating interferon activity in humans.

The siNA molecules described herein, the antisense region of a siNA molecule of the invention can comprise a phosphorothioate internucleotide linkage at the 3'-end of said antisense region. In any of the embodiments of siNA molecules described herein, the antisense region can comprise about one to about five phosphorothioate internucleotide linkages at the 5'-end of said antisense region. In any of the embodiments of siNA molecules described herein, the 3'-terminal nucleotide overhangs of a siNA molecule of the invention can comprise ribonucleotides or deoxyribonucleotides that are chemically-modified at a nucleic acid sugar, base, or backbone. In any of the embodiments of siNA molecules described herein, the 3'-terminal nucleotide overhangs can comprise one or more universal base ribonucleotides. In any of the embodiments of siNA molecules described herein, the 3'-terminal nucleotide overhangs can comprise one or more acyclic nucleotides.

For example, in a non-limiting example, the invention features a chemically-modified short interfering nucleic acid (siNA) having about 1, 2, 3, 4, 5, 6, 7, 8 or more phosphorothioate internucleotide linkages in one siNA strand. In yet another embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) individually having about 1, 2, 3, 4, 5, 6, 7, 8 or more phosphorothioate internucleotide linkages in both siNA strands. The phosphorothioate internucleotide linkages can be present in one or both oligonucleotide strands of the siNA duplex, for example in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more phosphorothioate internucleotide linkages at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (e.g., about 1, 2, 3, 4, 5, or more) consecutive phosphorothioate internucleotide linkages at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) pyrimidine phosphorothioate internucleotide linkages in the sense strand, the antisense strand, or both strands. In yet another

non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) purine phosphorothioate internucleotide linkages in the sense strand, the antisense strand, or both strands.

An siNA molecule may be comprised of a circular nucleic acid molecule, wherein the siNA is about 38 to about 70 (e.g., about 38, 40, 45, 50, 55, 60, 65, or 70) nucleotides in length having about 18 to about 23 (e.g., about 18, 19, 20, 21, 22, or 23) base pairs wherein the circular oligonucleotide forms a dumbbell shaped structure having about 19 base pairs and 2 loops.

A circular siNA molecule contains two loop motifs, wherein one or both loop portions of the siNA molecule is biodegradable. For example, a circular siNA molecule of the invention is designed such that degradation of the loop portions of the siNA molecule *in vivo* can generate a double-stranded siNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucleotides.

Modified nucleotides present in siNA molecules, preferably in the antisense strand of the siNA molecules, but also optionally in the sense and/or both antisense and sense strands, comprise modified nucleotides having properties or characteristics similar to naturally occurring ribonucleotides. For example, the invention features siNA molecules including modified nucleotides having a Northern conformation (e.g., Northern pseudorotation cycle, see for example, Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag ed., 1984). As such, chemically modified nucleotides present in the siNA molecules of the invention, preferably in the antisense strand of the siNA molecules of the invention, but also optionally in the sense and/or both antisense and sense strands, are resistant to nuclease degradation while at the same time maintaining the capacity to mediate RNAi. Non-limiting examples of nucleotides having a northern configuration include locked nucleic acid (LNA) nucleotides (e.g., 2'-O, 4'-C-methylene-(D-ribofuranosyl) nucleotides); 2'-methoxyethoxy (MOE) nucleotides; 2'-methyl-thio-ethyl, 2'-deoxy-2'-fluoro nucleotides. 2'-deoxy-2'-chloro nucleotides, 2'-azido nucleotides, and 2'-O-methyl nucleotides.

The sense strand of a double stranded siNA molecule may have a terminal cap moiety such as an inverted deoxybasic moiety, at the 3'-end, 5'-end, or both 3' and 5'-ends of the sense strand.

Non-limiting examples of conjugates include conjugates and ligands described in Vargeese, et al., U.S. Application Serial No. 10/427,160, filed April 30, 2003, incorporated by reference herein in its entirety, including the drawings. In another embodiment, the conjugate is covalently attached to the chemically-modified siNA molecule via a biodegradable linker. In one embodiment, the conjugate molecule is attached at both the 3'-end of either the sense strand,

the antisense strand, or both strands of the chemically-modified siNA molecule. In another embodiment, the conjugate molecule is attached at the 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In yet another embodiment, the conjugate molecule is attached at both the 3'-end and 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule, or any combination thereof. In one embodiment, a conjugate molecule of the invention comprises a molecule that facilitates delivery of a chemically-modified siNA molecule into a biological system, such as a cell. In another embodiment, the conjugate molecule attached to the chemically-modified siNA molecule is a polyethyleneglycol, human serum albumin, or a ligand for a cellular receptor that can mediate cellular uptake. Examples of specific conjugate molecules contemplated by the instant invention that can be attached to chemically-modified siNA molecules are described in Vargeese, et al., U.S. Patent Application Publication No. 20030130186, published July 10, 2003, and U.S. Patent Application Publication No. 20040110296, published June 10, 2004. The type of conjugates used and the extent of conjugation of siNA molecules of the invention can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of siNA constructs while at the same time maintaining the ability of the siNA to mediate RNAi activity. As such, one skilled in the art can screen siNA constructs that are modified with various conjugates to determine whether the siNA conjugate complex possesses improved properties while maintaining the ability to mediate RNAi, for example in animal models as are generally known in the art.

A siNA may be further comprised of a nucleotide, non-nucleotide, or mixed nucleotide/non-nucleotide linker that joins the sense region of the siNA to the antisense region of the siNA. In one embodiment, a nucleotide linker can be a linker of >2 nucleotides in length, for example about 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides in length. In another embodiment, the nucleotide linker can be a nucleic acid aptamer. By "aptamer" or "nucleic acid aptamer" as used herein is meant a nucleic acid molecule that binds specifically to a target molecule wherein the nucleic acid molecule has sequence that comprises a sequence recognized by the target molecule in its natural setting. Alternately, an aptamer can be a nucleic acid molecule that binds to a target molecule where the target molecule does not naturally bind to a nucleic acid. The target molecule can be any molecule of interest. For example, the aptamer can be used to bind to a ligand-binding domain of a protein, thereby preventing interaction of the naturally occurring ligand with the protein. This is a non-limiting example and those in the art will recognize that other embodiments can be readily generated using techniques generally known in the art. (See, for example, Gold, et al, *Annu. Rev. Biochem. 64*:763, 1995; Brody and Gold, *J. Biotechnol.*

74:5, 2000; Sun, *Curr. Opin. Mol. Ther. 2*:100, 2000; Kusser, *J. Biotechnol. 74*:27, 2000; Hermann and Patel, *Science 287*:820, 2000; and Jayasena, *Clinical Chemistry 45*:1628, 1999.

A non-nucleotide linker may be comprised of an abasic nucleotide, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, polyhydrocarbon, or other polymeric compounds (e.g., polyethylene glycols such as those having between 2 and 100 ethylene glycol units). Specific examples include those described by Seela and Kaiser, *Nucleic Acids Res. 18*:6353, 1990 and *Nucleic Acids Res. 15*:3113, 1987; Cload and Schepartz, *J. Am. Chem. Soc. 113*:6324, 1991; Richardson and Schepartz, *J. Am. Chem. Soc. 113*:5109, 1991; Ma, et al., *Nucleic Acids Res. 21*:2585, 1993 and *Biochemistry 32*:1751, 1993; Durand, et al., *Nucleic Acids Res. 18*:6353, 1990; McCurdy, et al., *Nucleosides & Nucleotides 10*:287, 1991; Jschke, et al., *Tetrahedron Lett. 34*:301, 1993; Ono, et al., *Biochemistry 30*:9914, 1991; Arnold, et al., International Publication No. WO 89/02439; Usman, et al., International Publication No. WO 95/06731; Dudycz, et al., International Publication No. WO 95/11910 and Ferentz and Verdine, *J. Am. Chem. Soc. 113*:4000, 1991. A "non-nucleotide" further means any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound can be abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymidine, for example at the C1 position of the sugar.

Administration of Compositions Comprising Polynucleotide Delivery-Enhancing Polypeptide/siNA conjugates

Delivery-enhancing polypeptide/siNA conjugates of the present disclosure may be used to treat diseases or conditions as discussed herein or as otherwise known in the art. To treat a particular disease or condition, the delivery-enhancing polypeptide/siNA conjugates of the present invention may be administered to a patient or may be administered to other appropriate cells evident to those skilled in the art, individually or in combination with one or more compound under conditions suitable for the treatment.

For example, the delivery-enhancing polypeptide/siNA conjugates described herein can be used in combination with other known treatments and/or therapeutic agents to treat a wide variety of conditions, particularly viral infections. Non-limiting examples of other therapeutic agents that can be readily combined with a the delivery-enhancing polypeptide/siNA conjugate of the present invention include, for example, enzymatic nucleic acid molecules; allosteric nucleic acid molecules; antisense, decoy, or aptamer nucleic acid molecules; antibodies such as

monoclonal antibodies; small molecules; and other organic and/or inorganic compounds including metals, salts and ions.

Thus, the present disclosure describes compositions comprising one or more delivery-enhancing polypeptide/siNA conjugate(s) in an acceptable carrier, such as a stabilizer, buffer, and the like. The delivery-enhancing polypeptide/siNA conjugate may be administered to a patient by any standard means, with or without stabilizers, buffers, and the like, to form a composition suitable for treatment. When it is desired to use a liposome delivery mechanism, standard protocols for formation of liposomes can be followed. The compositions of the present invention may also be formulated and used as tablets, capsules or elixirs for oral administration, suppositories for rectal administration, sterile solutions, and suspensions for injectable administration, either with or without other compounds known in the art. Delivery-enhancing polypeptide/siNA conjugate formulations include salts of the above compounds, e.g., acid addition salts such as salts of hydrochloric acid, hydrobromic acid, acetic acid, and benzene sulfonic acid.

Pharmaceutical compositions or formulations refer to compositions or formulations in a form suitable for administration, e.g., systemic administration, into a cell or patient such as a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation from reaching a target cell (i.e., a cell to which the negatively charged nucleic acid is desirable for delivery). For example, pharmaceutical compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms that prevent the composition or formulation from exerting its effect.

Individual reporter gene expression constructs may be co-transfected with one or more delivery-enhancing polypeptide/siNA conjugate. The capacity of a given delivery-enhancing polypeptide/siNA conjugate to reduce the expression level of each of the contemplated gene variants may be determined by comparing the measured reporter gene activity from cells transfected with and without the modified siNA.

Methods for the delivery of nucleic acid molecules are described in Akhtar, et al., *Trends Cell Bio. 2*:139, 1992; "Delivery Strategies for Antisense Oligonucleotide Therapeutics" (ed. Akhtar, 1995); Maurer, et al., *Mol. Membr. Biol. 16*:129-140, 1999; Hofland and Huang, *Handbook Exp. Pharmacol. 137*:165-192, 1999; and Lee, et al., *ACS Symp. Ser. 752*:184-192, 2000. Sullivan, et al., PCT WO 94/02595, further describes the general methods for delivery of enzymatic nucleic acid molecules. These protocols can be utilized for the delivery of virtually any nucleic acid molecule.

As used herein, the term "systemic administration" is meant to include *in vivo* systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include, without limitation: intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes expose the desired negatively charged polymers, for example, nucleic acids, to an accessible diseased tissue. The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size.

Nucleic acid molecules can be administered to cells by a variety of methods known to those of skill in the art, including, but not restricted to, encapsulation in liposomes; by iontophoresis; or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres; or by proteinaceous vectors (O'Hare and Normand, International PCT Publication No. WO 00/53722). Alternatively, the nucleic acid/vehicle combination may be locally delivered by direct injection or by use of an infusion pump. Direct injection of the nucleic acid molecules of the invention, whether subcutaneous, intramuscular, or intradermal, can take place using standard needle and syringe methodologies, or by needle-free technologies such as those described in Conry, et al., *Clin. Cancer Res.* *5*:2330-2337, 1999, and Barry, et al., International PCT Publication No. WO 99/31262.

The delivery-enhancing polypeptide/siNA conjugates disclosed herein can be used as pharmaceutical agents. Pharmaceutical agents prevent, modulate the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state in a patient.

As used herein, the phrase "pharmaceutically acceptable formulation" is meant to include compositions or formulations that allows for the effective distribution of the nucleic acid molecules of the instant invention in the physical location most suitable for their desired activity. Nonlimiting examples of agents suitable for formulation with the nucleic acid molecules of the instant invention include: P-glycoprotein inhibitors (such as Pluronic P85), which can enhance entry of drugs into the CNS (Jolliet-Riant and Tillement, *Fundam. Clin. Pharmacol. 13*:16-26, 1999); biodegradable polymers, such as poly (DL-lactide-coglycolide) microspheres for sustained release delivery after intracerebral implantation (Emerich, et al., *Cell Transplant 8*:47-58, 1999) (Alkermes, Inc., Cambridge, Mass.); and loaded nanoparticles, such as those made of polybutylcyanoacrylate, which can deliver drugs across the blood brain barrier and can alter neuronal uptake mechanisms (*Prog Neuropsychopharmacol Biol Psychiatry 23*:941-949, 1999).

The use of a liposome or other drug carrier comprising the compounds of the instant invention can potentially localize the drug, for example, in certain tissue types, such as the tissues of the reticular endothelial system (RES). A liposome formulation that can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach may provide enhanced delivery of the drug to target cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of abnormal cells, such as cancer cells.

Other non-limiting examples of delivery strategies for the nucleic acid molecules of the instant invention include material described in Boado, et al., *J. Pharm. Sci. 87*:1308-1315, 1998; Tyler, et al., *FEBS Lett. 421*:280-284, 1999; Pardridge, et al., *PNAS USA 92*:5592-5596, 1995; Boado, *Adv. Drug Delivery Rev. 15*:73-107, 1995; Aldrian-Herrada, et al., *Nucleic Acids Res. 26*:4910-4916, 1998; and Tyler, et al., *PNAS USA. 96*:7053-7058, 1999.

This disclosure also features the use of the composition comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). These formulations offer a method for increasing the accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug. Lasic, et al., *Chem. Rev. 95*:2601-2627, 1995; Ishiwata, et al., *Chem. Pharm. Bull. 43*:1005-1011, 1995. Such liposomes have been shown to accumulate selectively in tumors, presumably by extravasation and capture in the neovascularized target tissues. Lasic, et al., *Science 267*:1275-1276, 1995; Oku, et al., *Biochim. Biophys. Acta 1238*:86-90, 1995. The long-circulating liposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes which are known to accumulate in tissues of the MPS. Liu, et al., *J. Biol. Chem. 42*:24864-24870, 1995; Choi, et al., International PCT Publication No. WO 96/10391; Ansell, et al., International PCT Publication No. WO 96/10390; and Holland, et al., International PCT Publication No. WO 96/10392. Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, because of their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen.

The present disclosure also includes compositions prepared for storage or administration, which include a pharmaceutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., A.R. Gennaro ed., 1985. For example,

preservatives, stabilizers, dyes and flavoring agents may be provided. These include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. In addition, antioxidants and suspending agents may be used.

A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence of, or treat (alleviate a symptom to some extent, preferably all of the symptoms) a disease state. The pharmaceutically effective dose depends on the type of disease, the composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors that those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered dependent upon potency of the negatively charged polymer.

The present disclosure also includes compositions prepared for storage or administration that include a pharmaceutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Supplemental or complementary methods for delivery of nucleic acid molecules are described, for example, in Akhtar, et al., *Trends Cell Bio. 2*:139, 1992; *Delivery Strategies for Antisense Oligonucleotide Therapeutics*, ed. Akhtar, 1995; Maurer, et al., *Mol. Membr. Biol. 16*:129-140, 1999; Hofland and Huang, *Handb. Exp. Pharmacol. 137*:165-192, 1999; and Lee, et al., *ACS Symp. Ser. 752*:184-192, 2000. Sullivan, et al., International PCT Publication No. WO 94/02595, further describes general methods for delivery of enzymatic nucleic acid molecules. These protocols can be utilized to supplement or complement delivery of virtually any nucleic acid molecule contemplated within the invention.

Nucleic acid molecules and polynucleotide delivery-enhancing polypeptides can be administered to cells by a variety of methods known to those of skill in the art, including, but not restricted to, administration within formulations that comprise the siNA and polynucleotide delivery-enhancing polypeptide alone, or that further comprise one or more additional components, such as a pharmaceutically acceptable carrier, diluent, excipient, adjuvant, emulsifier, buffer, stabilizer, preservative, and the like. In certain embodiments, the siNA and/or the polynucleotide delivery-enhancing polypeptide can be encapsulated in liposomes, administered by iontophoresis, or incorporated into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, bioadhesive microspheres, or proteinaceous vectors (see e.g., O'Hare and Normand, International PCT Publication No. WO 00/53722). Alternatively, a nucleic acid/peptide/vehicle combination can be locally delivered by direct injection or by use of an infusion pump. Direct injection of the nucleic acid molecules of the invention, whether subcutaneous, intramuscular, or intradermal, can take place using standard

needle and syringe methodologies, or by needle-free technologies such as those described in Conry, et al., *Clin. Cancer Res. 5*:2330-2337, 1999 and Barry, et al., International PCT Publication No. WO 99/31262.

The compositions of the instant invention can be effectively employed as pharmaceutical agents. Pharmaceutical agents prevent, modulate the occurrence or severity of, or treat (alleviate one or more symptom(s) to a detectable or measurable extent) of a disease state or other adverse condition in a patient.

Thus within additional embodiments the invention provides pharmaceutical compositions and methods featuring the presence or administration of one or more polynucleic acid(s), typically one or more siNAs, combined, complexed, or conjugated with a polynucleotide delivery-enhancing polypeptide, optionally formulated with a pharmaceutically-acceptable carrier, such as a diluent, stabilizer, buffer, and the like.

The present invention satisfies additional objects and advantages by providing short interfering nucleic acid (siNA) molecules that modulate expression of genes associated with a particular disease state or other adverse condition in a subject. Typically, the siNA will target a gene that is expressed at an elevated level as a causal or contributing factor associated with the subject disease state or adverse condition. In this context, the siNA will effectively downregulate expression of the gene to levels that prevent, alleviate, or reduce the severity or recurrence of one or more associated disease symptoms. Alternatively, for various distinct disease models where expression of the target gene is not necessarily elevated as a consequence or sequel of disease or other adverse condition, down regulation of the target gene will nonetheless result in a therapeutic result by lowering gene expression (i.e., to reduce levels of a selected mRNA and/or protein product of the target gene). Alternatively, siNAs of the invention may be targeted to lower expression of one gene, which can result in upregulation of a "downstream" gene whose expression is negatively regulated by a product or activity of the target gene.

Within exemplary embodiments, the compositions and methods of the invention are useful as therapeutic tools to regulate expression of tumor necrosis factor–α (TNF-α) to treat or prevent symptoms of rheumatoid arthritis (RA). In this context the invention further provides compounds, compositions, and methods useful for modulating expression and activity of TNF-α by RNA interference (RNAi) using small nucleic acid molecules. In more detailed embodiments, the invention provides small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (mRNA), and short hairpin RNA (shRNA) molecules, and related methods, that are

effective for modulating expression of TNF-α and/or TNF-α genes to prevent or alleviate symptoms of RA in mammalian subjects. Within these and related therapeutic compositions and methods, the use of chemically-modified siNAs will often improve properties of the modified siNAs in comparison to properties of native siNA molecules, for example by providing increased resistance to nuclease degradation in vivo, and/or through improved cellular uptake. As can be readily determined according to the disclosure herein, useful siNAs having multiple chemical modifications will retain their RNAi activity. The siNA molecules of the instant invention thus provide useful reagents and methods for a variety of therapeutic, diagnostic, target validation, genomic discovery, genetic engineering, and pharmacogenomic applications.

This siNAs of the present invention may be administered in any form, for example transdermally or by local injection (e.g., local injection at sites of psoriatic plaques to treat psoriasis, or into the joints of patients afflicted with psoriatic arthritis or RA). In more detailed embodiments, the invention provides formulations and methods to administer therapeutically effective amounts of siNAs directed against of a mRNA of TNF-α, which effectively down-regulate the TNF-α RNA and thereby reduce or prevent one or more TNF-α-associated inflammatory condition(s). Comparable methods and compositions are provided that target expression of one or more different genes associated with a selected disease condition in animal subjects, including any of a large number of genes whose expression is known to be aberrantly increased as a causal or contributing factor associated with the selected disease condition.

The siNA/polynucleotide delivery-enhancing polypeptide mixtures of the invention can be administered in conjunction with other standard treatments for a targeted disease condition, for example in conjunction with therapeutic agents effective against inflammatory diseases, such as RA or psoriasis. Examples of combinatorially useful and effective agents in this context include non-steroidal anti-inflammatory drugs (NSAIDs), methotrexate, gold compounds, D-penicillamine, the antimalarials, sulfasalazine, glucocorticoids, and other TNF-α neutralizing agents such as infliximab and entracept.

Negatively charged polynucleotides of the invention (e.g., RNA or DNA) can be administered to a patient by any standard means, with or without stabilizers, buffers, and the like, to form a pharmaceutical composition. When it is desired to use a liposome delivery mechanism, standard protocols for formation of liposomes can be followed. The compositions of the present invention may also be formulated and used as tablets, capsules or elixirs for oral administration, suppositories for rectal administration, sterile solutions, suspensions for injectable administration, and the other compositions known in the art.

The present invention also includes pharmaceutically acceptable formulations of the compositions described herein. These formulations include salts of the above compounds, e.g., acid addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid.

A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, e.g., systemic administration, into a cell or patient, including for example a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation from reaching a target cell (i.e., a cell to which the negatively charged nucleic acid is desirable for delivery). For example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity.

By "systemic administration" is meant in vivo systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include, without limitation: intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes exposes the desired negatively charged polymers, e.g., nucleic acids, to an accessible diseased tissue. The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier comprising the compounds of the instant invention can potentially localize the drug, for example, in certain tissue types, such as the tissues of the reticular endothelial system (RES). A liposome formulation that can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach may provide enhanced delivery of the drug to target cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of abnormal cells, such as cancer cells.

By "pharmaceutically acceptable formulation" is meant, a composition or formulation that allows for the effective distribution of the nucleic acid molecules of the instant invention in the physical location most suitable for their desired activity. Nonlimiting examples of agents suitable for formulation with the nucleic acid molecules of the instant invention include: P-glycoprotein inhibitors (such as Pluronic P85), which can enhance entry of drugs into the CNS (Jolliet-Riant and Tillement, *Fundam. Clin. Pharmacol. 13*:16-26, *1999*); biodegradable polymers, such as poly (DL-lactide-coglycolide) microspheres for sustained release delivery after intracerebral implantation (Emerich, D.F., et al., *Cell Transplant 8*:47-58, 1999) (Alkermes, Inc. Cambridge, Mass.); and loaded nanoparticles, such as those made of

polybutylcyanoacrylate, which can deliver drugs across the blood brain barrier and can alter neuronal uptake mechanisms (*Prog. Neuropsychopharmacol Biol. Psychiatry 23*:941-949, 1999). Other non-limiting examples of delivery strategies for the nucleic acid molecules of the instant invention include material described in Boado, et al., *J. Pharm. Sci. 87*:1308-1315, 1998; Tyler, et al., *FEBS Lett. 421*:280-284, 1999; Pardridge, et al., *PNAS USA. 92*:5592-5596, 1995; Boado, *Adv. Drug Delivery Rev. 15*:73-107, 1995; Aldrian-Herrada, et al., *Nucleic Acids Res. 26*:4910-4916, 1998; and Tyler, et al., *PNAS USA. 96*:7053-7058, 1999.

The present invention also includes compositions prepared for storage or administration, which include a pharmaceutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., A.R. Gennaro ed., 1985, hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents may be provided. These include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. In addition, antioxidants and suspending agents may be used.

A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence of, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state. The pharmaceutically effective dose depends on the type of disease, the composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors that those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered dependent upon potency of the negatively charged polymer.

The delivery-enhancing polypeptide/siNA conjugates of the disclosure and formulations thereof can be administered orally, topically, parenterally, by inhalation or spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and/or vehicles. The term parenteral as used herein includes percutaneous, subcutaneous, intravascular (e.g., intravenous), intramuscular, or intrathecal injection or infusion techniques and the like. In addition, there is provided a pharmaceutical formulation comprising a nucleic acid molecule of the invention and a pharmaceutically acceptable carrier. One or more nucleic acid molecules of the invention can be present in association with one or more non-toxic pharmaceutically acceptable carriers and/or diluents and/or adjuvants, and if desired other active ingredients. The pharmaceutical compositions containing nucleic acid molecules of the invention can be in a form suitable for oral use, for example, as tablets, troches, lozenges,

aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs.

Compositions intended for oral use can be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions can contain one or more such sweetening agents, flavoring agents, coloring agents or preservative agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients that are suitable for the manufacture of tablets. These excipients can be, for example, inert diluents; such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, corn starch, or alginic acid; binding agents, for example starch, gelatin or acacia; and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets can be uncoated or they can be coated by known techniques. In some cases such coatings can be prepared by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monosterate or glyceryl distearate can be employed.

Formulations for oral use can also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example peanut oil, liquid paraffin or olive oil.

Aqueous suspensions contain the active materials in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydropropyl-methylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents can be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions can also contain one or more preservatives, for example ethyl, or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose or saccharin.

Oily suspensions can be formulated by suspending the active ingredients in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions can contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents and flavoring agents can be added to provide palatable oral preparations. These compositions can be preserved by the addition of an anti-oxidant such as ascorbic acid.

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents or suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavoring and coloring agents, can also be present.

Pharmaceutical compositions of the invention can also be in the form of oil-in-water emulsions. The oily phase can be a vegetable oil or a mineral oil or mixtures of these. Suitable emulsifying agents can be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally-occurring phosphatides, for example soy bean, lecithin, and esters or partial esters derived from fatty acids and hexitol, anhydrides, for example sorbitan monooleate, and condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monooleate. The emulsions can also contain sweetening and flavoring agents.

Syrups and elixirs can be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol, glucose or sucrose. Such formulations can also contain a demulcent, a preservative and flavoring, and coloring agents. The pharmaceutical compositions can be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension can be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents that have been mentioned above. The sterile injectable preparation can also be a sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil can be employed including synthetic mono-or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

The delivery-enhancing polypeptide/siNA conjugates disclosed hereinsiNAs can also be administered in the form of suppositories, e.g., for rectal administration of the drug. These compositions can be prepared by mixing the drug with a suitable non-irritating excipient that is

solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Such materials include cocoa butter and polyethylene glycols.

Delivery-enhancing polypeptide/siNA conjugates disclosed herein can be administered parenterally in a sterile medium. The drug, depending on the vehicle and concentration used, can either be suspended or dissolved in the vehicle. Advantageously, adjuvants such as local anesthetics, preservatives and buffering agents can be dissolved in the vehicle.

Dosage levels of the order of from about 0.1 mg to about 140 mg per kilogram of body weight per day are useful in the treatment of the above-indicated conditions (about 0.5 mg to about 7 g per patient per day). The amount of active ingredient that can be combined with the carrier materials to produce a single dosage form varies depending upon the host treated and the particular mode of administration. Dosage unit forms generally contain between from about 1 mg to about 500 mg of an active ingredient.

It is understood that the specific dose level for any particular patient depends upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, and rate of excretion, drug combination and the severity of the particular disease undergoing therapy.

For administration to non-human animals, the composition can also be added to the animal feed or drinking water. It can be convenient to formulate the animal feed and drinking water compositions so that the animal takes in a therapeutically appropriate quantity of the composition along with its diet. It can also be convenient to present the composition as a premix for addition to the feed or drinking water.

Delivery-enhancing polypeptide/siNA conjugates may also be administered to a patient in combination with other therapeutic compounds to increase the overall therapeutic effect. The use of multiple compounds to treat an indication may increase the beneficial effects while reducing the presence of side effects.

In one embodiment, the inventive compositions suitable for administering delivery-enhancing polypeptide/siNA conjugates to specific cell types, such as hepatocytes. For example, the asialoglycoprotein receptor (ASGPr) is unique to hepatocytes and binds branched galactose-terminal glycoproteins, such as asialoorosomucoid (ASOR). WuThe siNAs can be modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, 2'-O-methyl, 2'-H. (For a review see Usman and Cedergren, *TIBS 17*:34, 1992; Usman, et al., *Nucleic Acids Symp. Ser. 31*:163, 1994). SiNA constructs can be purified by gel electrophoresis using general methods or can be purified by high pressure liquid chromatography and re-suspended in water.

Chemically synthesizing nucleic acid molecules with modifications (base, sugar and/or phosphate) can prevent their degradation by serum ribonucleases, which can increase their potency. See e.g., Eckstein, et al., International Publication No. WO 92/07065; Perrault, et al., *Nature 344*:565, 1990; Pieken, et al., *Science 253*:314, 1991; Usman and Cedergren, *Trends in Biochem. Sci. 17*:334, 1992; Usman, et al., International Publication No. WO 93/15187; and Rossi, et al., International Publication No. WO 91/03162; Sproat, U.S. Patent No. 5,334,711; Gold, et al., U.S. Patent No. 6,300,074. All of the above references describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of the nucleic acid molecules described herein.

There are several examples in the art describing sugar, base and phosphate modifications that can be introduced into nucleic acid molecules with significant enhancement in their nuclease stability and efficacy. For example, oligonucleotides are modified to enhance stability and/or enhance biological activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, 2'-O-methyl, 2'-O-allyl, 2'-H, nucleotide base modifications. For a review see Usman and Cedergren, *TIBS 17*:34, 1992; Usman, et al., *Nucleic Acids Symp. Ser. 31*:163, 1994; Burgin, et al., *Biochemistry 35*:14090, 1996. Sugar modification of nucleic acid molecules have been extensively described in the art. See Eckstein, et al., International Publication PCT No. WO 92/07065; Perrault, et al., *Nature 344*:565-568, 1990; Pieken, et al., *Science 253*:314-317, 1991; Usman and Cedergren, *Trends in Biochem. Sci. 17*:334-339, 1992; Usman, et al., International Publication PCT No. WO 93/15187; Sproat, U.S. Patent No. 5,334,711 and Beigelman, et al., *J. Biol. Chem. 270*:25702, 1995; Beigelman, et al., International PCT Publication No. WO 97/26270; Beigelman, et al., U.S. Patent No. 5,716,824; Usman, et al., U.S. Patent No. 5,627,053; Woolf, et al., International PCT Publication No. WO 98/13526; Thompson, et al., Karpeisky, et al., *Tetrahedron Lett. 39*:1131, 1998; Earnshaw and Gait, *Biopolymers (Nucleic Acid Sciences) 48*:39-55, 1998; Verma and Eckstein, *Annu. Rev. Biochem. 67*:99-134, 1998; and Burlina, et al., *Bioorg. Med. Chem. 5*:1999-2010, 1997. Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into nucleic acid molecules without modulating catalysis. In view of such teachings, similar modifications can be used as described herein to modify the siNA nucleic acid molecules of the instant invention so long as the ability of siNA to promote RNAi in cells is not significantly inhibited.

While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorodithioate, and/or 5'-methylphosphonate linkages improves stability, excessive modifications can cause some toxicity or decreased activity. Therefore, when

designing nucleic acid molecules, the amount of these internucleotide linkages should be minimized. The reduction in the concentration of these linkages should lower toxicity, resulting in increased efficacy and Wu, *J. Biol. Chem. 262*:4429-4432, 1987. Binding of such glycoproteins or synthetic glycoconjugates to the receptor takes place with an affinity that strongly depends on the degree of branching of the oligosaccharide chain, for example, triatennary structures are bound with greater affinity than biatenarry or monoatennary chains. Baenziger and Fiete, *Cell 22*:611-620, 1980, and Connolly, et al., *J. Biol. Chem. 257*:939-945, 1982. Lee and Lee obtained this high specificity through the use of N-acetyl-D-galactosamine as the carbohydrate moiety, which has higher affinity for the receptor, compared to galactose. *Glycoconjugate J. 4*:317-328, 1987. This "clustering effect" has also been described for the binding and uptake of mannosyl-terminating glycoproteins or glycoconjugates. Ponpipom, et al., *J. Med. Chem. 24*:1388-1395, 1981. The use of galactose and galactosamine nucleotided conjugates to transport exogenous compounds across cell membranes can provide a targeted delivery approach to the treatment of liver disease such as HBV infection or hepatocellular carcinoma. The use of bioconjugates can also provide a reduction in the required dose of therapeutic compounds required for treatment. Furthermore, therapeutic bioavialability, pharmacodynamics, and pharmacokinetic parameters can be modulated through the use of nucleic acid bioconjugates of the invention.higher specificity of these molecules.

In one embodiment, the invention features modified siNA molecules, with phosphate backbone modifications comprising one or more phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and/or alkylsilyl, substitutions. For a review of oligonucleotide backbone modifications, see Hunziker and Leumann, "Nucleic Acid Analogues: Synthesis and Properties, in Modern Synthetic Methods," *VCH*, 331-417, 1995, and Mesmaeker, et al., "Novel Backbone Replacements for Oligonucleotides, in Carbohydrate Modifications in Antisense Research," *ACS*, 24-39, 1994.

Methods for the delivery of nucleic acid molecules are described in Akhtar, et al., *Trends Cell Bio. 2:*139, 1992; Delivery Strategies for Antisense Oligonucleotide Therapeutics, ed. Akhtar, 1995; Maurer, et al., *Mol. Membr. Biol. 16*:129-140, 1999; Hofland and Huang, *Handb. Exp. Pharmacol. 137*:165-192, 1999; and Lee, et al., *ACS Symp. Ser. 752*:184-192, 2000. Beigelman, et al., U.S. Patent No. 6,395,713 and Sullivan, et al., PCT WO 94/02595 further describe the general methods for delivery of nucleic acid molecules. These protocols can be utilized for the delivery of virtually any nucleic acid molecule. Nucleic acid molecules can be administered to cells by a variety of methods known to those of skill in the art, including, but not

restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as biodegradable polymers, hydrogels, cyclodextrins (see for example, Gonzalez, et al., *Bioconjugate Chem. 10*:1068-1074, 1999; Wang, et al., International PCT Publication Nos. WO 03/47518 and WO 03/46185), poly(lactic-co-glycolic)ac- id (PLGA) and PLCA microspheres (see for example, U.S. Patent No. 6,447,796 and U.S. Patent Application Publication No. US 2002130430), biodegradable nanocapsules, and bioadhesive microspheres, or by proteinaceous vectors (O'Hare and Normand, International PCT Publication No. WO 00/53722). Alternatively, the nucleic acid/vehicle combination is locally delivered by direct injection or by use of an infusion pump. Direct injection of the nucleic acid molecules of the invention, whether subcutaneous, intramuscular, or intradermal, can take place using standard needle and syringe methodologies, or by needle-free technologies such as those described in Conry, et al., *Clin. Cancer Res. 5*:2330-2337, 1999, and Barry, et al., International PCT Publication No. WO 99/31262. The molecules of the instant invention can be used as pharmaceutical agents. Pharmaceutical agents prevent, modulate the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state in a subject.

The term "ligand" refers to any compound or molecule, such as a drug, peptide, hormone, or neurotransmitter, that is capable of interacting with another compound, such as a receptor, either directly or indirectly. The receptor that interacts with a ligand can be present on the surface of a cell or can alternately be an intracellular receptor. Interaction of the ligand with the receptor can result in a biochemical reaction, or can simply be a physical interaction or association.

By "asymmetric hairpin" as used herein is meant a linear siNA molecule comprising an antisense region, a loop portion that can comprise nucleotides or non-nucleotides, and a sense region that comprises fewer nucleotides than the antisense region to the extent that the sense region has enough complementary nucleotides to base pair with the antisense region and form a duplex with loop. For example, an asymmetric hairpin siNA molecule of the invention can comprise an antisense region having length sufficient to mediate RNAi in a T-cell (e.g., about 19 to about 22 (e.g., about 19, 20, 21, or 22) nucleotides) and a loop region comprising about 4 to about 8 (e.g., about 4, 5, 6, 7, or 8) nucleotides, and a sense region having about 3 to about 18 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18) nucleotides that are complementary to the antisense region. The asymmetric hairpin siNA molecule can also comprise a 5'-terminal phosphate group that can be chemically modified. The loop portion of the

asymmetric hairpin siNA molecule can comprise nucleotides, non-nucleotides, linker molecules, or conjugate molecules as described herein.

By "asymmetric duplex" as used herein is meant a siNA molecule having two separate strands comprising a sense region and an antisense region, wherein the sense region comprises fewer nucleotides than the antisense region to the extent that the sense region has enough complementary nucleotides to base pair with the antisense region and form a duplex. For example, an asymmetric duplex siNA molecule of the invention can comprise an antisense region having length sufficient to mediate RNAi in a T-cell (e.g., about 19 to about 22 (e.g., about 19, 20, 21, or 22) nucleotides) and a sense region having about 3 to about 18 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18) nucleotides that are complementary to the antisense region.

By "modulate gene expression" is meant that the expression of a target gene is upregulated or downregulated, which can include upregulation or downregulation of mRNA levels present in a cell, or of mRNA translation, or of synthesis of protein or protein subunits, encoded by the target gene. Modulation of gene expression can be determined also be the presence, quantity, or activity of one or more proteins or protein subunits encoded by the target gene that is up regulated or down regulated, such that expression, level, or activity of the subject protein or subunit is greater than or less than that which is observed in the absence of the modulator (e.g., a siRNA). For example, the term "modulate" can mean "inhibit," but the use of the word "modulate" is not limited to this definition.

By "inhibit", "down-regulate", or "reduce" expression, it is meant that the expression of the gene, or level of RNA molecules or equivalent RNA molecules encoding one or more proteins or protein subunits, or level or activity of one or more proteins or protein subunits encoded by a target gene, is reduced below that observed in the absence of the nucleic acid molecules (e.g., siNA) of the invention. In one embodiment, inhibition, down-regulation or reduction with an siNA molecule is below that level observed in the presence of an inactive or attenuated molecule. In another embodiment, inhibition, down-regulation, or reduction with siNA molecules is below that level observed in the presence of, for example, an siNA molecule with scrambled sequence or with mismatches. In another embodiment, inhibition, down-regulation, or reduction of gene expression with a nucleic acid molecule of the instant invention is greater in the presence of the nucleic acid molecule than in its absence.

Gene "silencing" refers to partial or complete loss-of-function through targeted inhibition of gene expression in a cell and may also be referred to as "knock down." Depending on the circumstances and the biological problem to be addressed, it may be preferable to partially

reduce gene expression. Alternatively, it might be desirable to reduce gene expression as much as possible. The extent of silencing may be determined by methods known in the art, some of which are summarized in International Publication No. WO 99/32619. Depending on the assay, quantification of gene expression permits detection of various amounts of inhibition that may be desired in certain embodiments of the invention, including prophylactic and therapeutic methods, which will be capable of knocking down target gene expression, in terms of mRNA levels or protein levels or activity, for example, by equal to or greater than 10%, 30%, 50%, 75% 90%, 95% or 99% of baseline (i.e., normal) or other control levels, including elevated expression levels as may be associated with particular disease states or other conditions targeted for therapy.

The phrase "inhibiting expression of a target gene" refers to the ability of a siNA of the invention to initiate gene silencing of the target gene. To examine the extent of gene silencing, samples or assays of the organism of interest or cells in culture expressing a particular construct are compared to control samples lacking expression of the construct. Control samples (lacking construct expression) are assigned a relative value of 100%. Inhibition of expression of a target gene is achieved when the test value relative to the control is about 90%, often 50%, and in certain embodiments 25-0%. Suitable assays include, e.g., examination of protein or mRNA levels using techniques known to those of skill in the art such as dot blots, northern blots, in situ hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

By "subject" is meant an organism, tissue, or cell, which may include an organism as the subject or as a donor or recipient of explanted cells or the cells that are themselves subjects for siNA delivery. "Subject" therefore may refers to an organism, organ, tissue, or cell, including *in vitro* or *ex vivo* organ, tissue or cellular subjects, to which the nucleic acid molecules of the invention can be administered and enhanced by polynucleotide delivery-enhancing polypeptides described herein. Exemplary subjects include mammalian individuals or cells, for example human patients or cells.

As used herein "cell" is used in its usual biological sense, and does not refer to an entire multicellular organism, e.g., specifically does not refer to a human. The cell can be present in an organism, e.g., birds, plants and mammals such as humans, cows, sheep, apes, monkeys, swine, dogs, and cats. The cell can be prokaryotic (e.g., bacterial cell) or eukaryotic (e.g., mammalian or plant cell). The cell can be of somatic or germ line origin, totipotent or pluripotent, dividing or non-dividing. The cell can also be derived from or can comprise a gamete or embryo, a stem cell, or a fully differentiated cell.

By "vectors" is meant any nucleic acid- and/or viral-based technique used to deliver a desired nucleic acid.

By "comprising" is meant including, but not limited to, whatever follows the word "comprising." Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of." Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

By "RNA" is meant a molecule comprising at least one ribonucleotide residue. By "ribonucleotide" is meant a nucleotide with a hydroxyl group at the 2' position of a .beta.-D-ribo-furanose moiety. The term "RNA" includes double-stranded RNA, single-stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the siNA or internally, for example at one or more nucleotides of the RNA. Nucleotides in the RNA molecules of this invention can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of naturally-occurring RNA.

By "highly conserved sequence region" is meant, a nucleotide sequence of one or more regions in a target gene does not vary significantly from one generation to the other or from one biological system to the other.

By "sense region" is meant a nucleotide sequence of a siNA molecule having complementarity to an antisense region of the siNA molecule. In addition, the sense region of a siNA molecule can comprise a nucleic acid sequence having homology with a target nucleic acid sequence.

By "antisense region" is meant a nucleotide sequence of a siNA molecule having complementarity to a target nucleic acid sequence. In addition, the antisense region of a siNA

molecule can optionally comprise a nucleic acid sequence having complementarity to a sense region of the siNA molecule.

By "target nucleic acid" is meant any nucleic acid sequence whose expression or activity is to be modulated. The target nucleic acid can be DNA or RNA.

By "complementarity" is meant that a nucleic acid can form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non-traditional types. In reference to the nucleic molecules of the present invention, the binding free energy for a nucleic acid molecule with its complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, e.g., RNAi activity. Determination of binding free energies for nucleic acid molecules is well known in the art (see, e.g., Turner, et al., *CSH Symp. Quant. Biol. LII*, 1987, pp. 123-133; Frier, et al., *Proc. Nat. Acad. Sci. USA 83*:9373-9377, 1986; Turner, et al., *J. Am. Chem. Soc. 109*:3783-3785, 1987). A percent complementarity indicates the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, or 10 nucleotides out of a total of 10 nucleotides in the first oligonucleotide being based paired to a second nucleic acid sequence having 10 nucleotides represents 50%, 60%, 70%, 80%, 90%, and 100% complementary respectively). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence.

The term "universal base" as used herein refers to nucleotide base analogs that form base pairs with each of the natural DNA/RNA bases with little discrimination between them. Non-limiting examples of universal bases include C-phenyl, C-naphthyl and other aromatic derivatives, inosine, azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole as known in the art (see for example, Loakes, *Nucleic Acids Research 29*:2437-2447, 2001).

The term "acyclic nucleotide" as used herein refers to any nucleotide having an acyclic ribose sugar, for example where any of the ribose carbons (C1, C2, C3, C4, or C5), are independently or in combination absent from the nucleotide.

The term "biodegradable" as used herein, refers to degradation in a biological system, for example enzymatic degradation or chemical degradation.

The term "biologically active molecule" as used herein, refers to compounds or molecules that are capable of eliciting or modifying a biological response in a system. Non-limiting examples of biologically active siNA molecules either alone or in combination with other molecules contemplated by the instant invention include therapeutically active molecules such as

antibodies, cholesterol, hormones, antivirals, peptides, proteins, chemotherapeutics, small molecules, vitamins, co-factors, nucleosides, nucleotides, oligonucleotides, enzymatic nucleic acids, antisense nucleic acids, triplex forming oligonucleotides, 2,5-A chimeras, siNA, dsRNA, allozymes, aptamers, decoys and analogs thereof. Biologically active molecules of the invention also include molecules capable of modulating the pharmacokinetics and/or pharmacodynamics of other biologically active molecules, for example, lipids and polymers such as polyamines, polyamides, polyethylene glycol and other polyethers.

The term "phospholipid" as used herein, refers to a hydrophobic molecule comprising at least one phosphorus group. For example, a phospholipid can comprise a phosphorus-containing group and saturated or unsaturated alkyl group, optionally substituted with OH, COOH, oxo, amine, or substituted or unsubstituted aryl groups.

By "cap structure" is meant chemical modifications, which have been incorporated at either terminus of the oligonucleotide (see, for example, Adamic, et al., U.S. Patent No. 5,998,203, incorporated by reference herein). These terminal modifications protect the nucleic acid molecule from exonuclease degradation, and may help in delivery and/or localization within a cell. The cap may be present at the 5'-terminus (5'-cap) or at the 3'-terminal (3'-cap) or may be present on both termini. In non-limiting examples, the 5'-cap includes, but is not limited to, glyceryl, inverted deoxy abasic residue (moiety); 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide, 4'-thio nucleotide; carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; threo-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; or bridging or non-bridging methylphosphonate moiety.

Non-limiting examples of the 3'-cap include, but are not limited to, glyceryl, inverted deoxy abasic residue (moiety), 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate; 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; threo-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moiety; 5'-5'-inverted abasic moiety; 5'-phosphoramidate;

5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties (for more details see Beaucage and Lyer, *Tetrahedron 49*:1925, 1993; incorporated by reference herein).

By the term "non-nucleotide" is meant any group or compound which can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine and therefore lacks a base at the 1'-position.

By "nucleotide" as used herein is as recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a nucleotide sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other; see, for example, Usman and McSwiggen, *supra*; Eckstein, et al., International PCT Publication No. WO 92/07065; Usman, et al., International PCT Publication No. WO 93/15187; Uhlman & Peyman, *supra*, all are hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art as summarized by Limbach, et al., *Nucleic Acids Res. 22*:2183, 1994. Some of the non-limiting examples of base modifications that can be introduced into nucleic acid molecules include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (e.g., 5-methylcytidine), 5-alkyluridines (e.g., ribothymidine), 5-halouridine (e.g., 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (e.g. 6-methyluridine), propyne, and others (Burgin, et al., *Biochemistry 35*:14090, 1996; Uhlman & Peyman, *supra*). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents.

By "target site" is meant a sequence within a target RNA that is "targeted" for cleavage mediated by a siNA construct which contains sequences within its antisense region that are complementary to the target sequence.

By "detectable level of cleavage" is meant cleavage of target RNA (and formation of cleaved product RNAs) to an extent sufficient to discern cleavage products above the background of RNAs produced by random degradation of the target RNA. Production of

cleavage products from 1-5% of the target RNA is sufficient to detect above the background for most methods of detection.

By "biological system" is meant, material, in a purified or unpurified form, from biological sources, including but not limited to human, animal, plant, insect, bacterial, viral or other sources, wherein the system comprises the components required for RNAi activity. The term "biological system" includes, for example, a cell, tissue, or organism, or extract thereof. The term biological system also includes reconstituted RNAi systems that can be used in an *in vitro* setting.

The term "biodegradable linker" as used herein, refers to a nucleic acid or non-nucleic acid linker molecule that is designed as a biodegradable linker to connect one molecule to another molecule, for example, a biologically active molecule to a siNA molecule of the invention or the sense and antisense strands of a siNA molecule of the invention. The biodegradable linker is designed such that its stability can be modulated for a particular purpose, such as delivery to a particular tissue or cell type. The stability of a nucleic acid-based biodegradable linker molecule can be modulated by using various chemistries, for example combinations of ribonucleotides, deoxyribonucleotides, and chemically-modified nucleotides, such as 2'-O-methyl, 2'-fluoro, 2'-amino, 2'-O-amino, 2'-C-allyl, 2'-O-allyl, and other 2'-modified or base modified nucleotides. The biodegradable nucleic acid linker molecule can be a dimer, trimer, tetramer or longer nucleic acid molecule, for example, an oligonucleotide of about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length, or can comprise a single nucleotide with a phosphorus-based linkage, for example, a phosphoramidate or phosphodiester linkage. The biodegradable nucleic acid linker molecule can also comprise nucleic acid backbone, nucleic acid sugar, or nucleic acid base modifications.

By "abasic" is meant sugar moieties lacking a base or having other chemical groups in place of a base at the 1' position, see for example Adamic, et al., U.S. Patent No. 5,998,203.

By "unmodified nucleoside" is meant one of the bases adenine, cytosine, guanine, thymine, or uracil joined to the 1' carbon of beta.-D-ribo-furanose.

By "modified nucleoside" is meant any nucleotide base which contains a modification in the chemical structure of an unmodified nucleotide base, sugar and/or phosphate. Non-limiting examples of modified nucleotides are shown by Formulae I-VII and/or other modifications described herein.

In connection with 2'-modified nucleotides as described for the present invention, by "amino" is meant 2'-NH_2 or 2'-O--NH_2, which can be modified or unmodified. Such modified

groups are described, for example, in Eckstein, et al., U.S. Patent No. 5,672,695 and Matulic-Adamic, et al., U.S. Patent No. 6,248,878.

The siNA molecules can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells or tissues. The nucleic acid or nucleic acid complexes can be locally administered to through injection, infusion pump or stent, with or without their incorporation in biopolymers. In another embodiment, polyethylene glycol (PEG) can be covalently attached to siNA compounds of the present invention, to the polynucleotide delivery-enhancing polypeptide, or both. The attached PEG can be any molecular weight, preferably from about 2,000 to about 50,000 daltons (Da).

The sense region can be connected to the antisense region via a linker molecule, such as a polynucleotide linker or a non-nucleotide linker.

"Inverted repeat" refers to a nucleic acid sequence comprising a sense and an antisense element positioned so that they are able to form a double stranded siRNA when the repeat is transcribed. The inverted repeat may optionally include a linker or a heterologous sequence such as a self-cleaving ribozyme between the two elements of the repeat. The elements of the inverted repeat have a length sufficient to form a double stranded RNA. Typically, each element of the inverted repeat is about 15 to about 100 nucleotides in length, preferably about 20-30 base nucleotides, preferably about 20-25 nucleotides in length, e.g., 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

"Nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

"Large double-stranded RNA" refers to any double-stranded RNA having a size greater than about 40 base pairs (bp) for example, larger than 100 bp or more particularly larger than 300 bp. The sequence of a large dsRNA may represent a segment of a mRNA or the entire mRNA. The maximum size of the large dsRNA is not limited herein. The double-stranded RNA may include modified bases where the modification may be to the phosphate sugar backbone or to the nucleoside. Such modifications may include a nitrogen or sulfur heteroatom or any other modification known in the art.

The double-stranded structure may be formed by self-complementary RNA strand such as occurs for a hairpin or a micro RNA or by annealing of two distinct complementary RNA strands.

"Overlapping" refers to when two RNA fragments have sequences which overlap by a plurality of nucleotides on one strand, for example, where the plurality of nucleotides (nt) numbers as few as 2-5 nucleotides or by 5-10 nucleotides or more.

"One or more dsRNAs" refers to dsRNAs that differ from each other on the basis of sequence.

"Target gene or mRNA" refers to any gene or mRNA of interest. Indeed any of the genes previously identified by genetics or by sequencing may represent a target. Target genes or mRNA may include developmental genes and regulatory genes as well as metabolic or structural genes or genes encoding enzymes. The target gene may be expressed in those cells in which a phenotype is being investigated or in an organism in a manner that directly or indirectly impacts a phenotypic characteristic. The target gene may be endogenous or exogenous. Such cells include any cell in the body of an adult or embryonic animal or plant including gamete or any isolated cell such as occurs in an immortal cell line or primary cell culture.

In this specification and the appended claims, the singular forms of "a", "an" and "the" include plural reference unless the context clearly dictates otherwise.

The examples given herein are solely for the purpose of illustration and are not intended to limit the scope of the invention as described in the claims. Although specific terms and values have been employed herein, such terms and values will be understood as exemplary and non to limit the scope of the invention.

All publications and references cited in this disclosure are hereby incorporated by reference in their entirety for all purposes.

EXAMPLES

The above disclosure generally describes the present invention, which is further exemplified by the following examples. These examples are described solely for purposes of illustration, and are not intended to limit the scope of the invention. Although specific terms and values have been employed herein, such terms and values will likewise be understood as exemplary and non-limiting to the scope of the invention.

Example 1

Protocols and Methods Used

Synthesis of siRNA/Polypeptide Conjugates

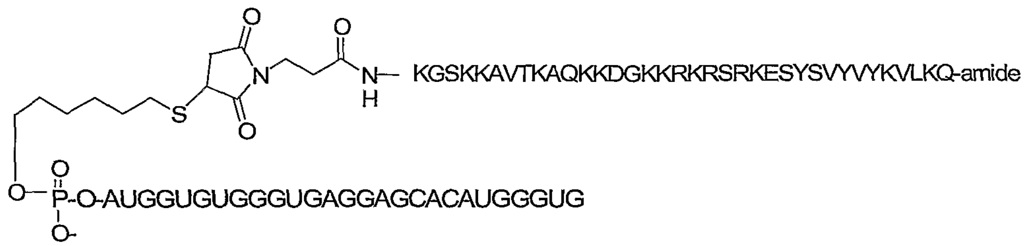
Both polypeptides and RNA are prepared using standard solid phase synthesis methods. The polypeptide and RNA molecules must be functionalized with specific moieties to allow for covalent attachment to each other. For the polypeptide, the N-terminus is functionalized with 3-maleimidopropionic acid. However, it is recognized that other functional groups such as bromo or iodoacetyl moieties will work as well. For the RNA molecule 5' end of the sense strand or 5' end of the antisense strand is functionalized with a 1-O-dimethoxytrityl-hexyl-disulfide linker.

The RNA oligo was dissolved in 0.5 mL of water to which was added 2 mL of buffer A (20 mM Tris-HCl, pH 6.8, 50% formamide). The polypeptide PN277 was subsequently added which resulted in the formation of a precipitate that was solubilized by the additional 1 mL of 2 M TEAA buffer. Upon completion of the reaction, the solvent was removed under reduced pressure and the resulting solid was dissolved in buffer A (20 mM Tris-HCl, pH 6.8, 50% formamide). The material was loaded onto an Amersham Resource Q column and washed with 5 column volumes of buffer A at 6 mL/min. Separation was accomplished by running a linear gradient of Buffer B (20 mM Tris-HCl, pH 6.8, 50% formamide, 1 M NaCl) from 15-60% for 15 column volumes at a flow rate of 6 mL/min. The purified conjugate was desalted by slidalyzer dialysis cassettes (3.5 K MWCO) against PBS. The amount of the conjugate was determined spectrophotometrically based on the calculated molar absorption coefficient at λ=260 nm. Analysis of the purity of this conjugate by RP-HPLC is shown below.

The anti-sense RNA strand conjugated to the polypeptide was annealed to its complimentary sense RNA strand in 50 mM potassium acetate, 1 mM magnesium acetate and 15 mM HEPES pH 7.4 and heating at 90°C for two minutes followed by incubation at 37^0C for one hour. The formation of the double stranded RNA conjugate was confirmed by non denaturing (15%) polyacrylamide gel electrophoresis and ethidium bromide staining.

An example of an siRNA/polypeptide conjugate of the present invention is shown in Example 3.

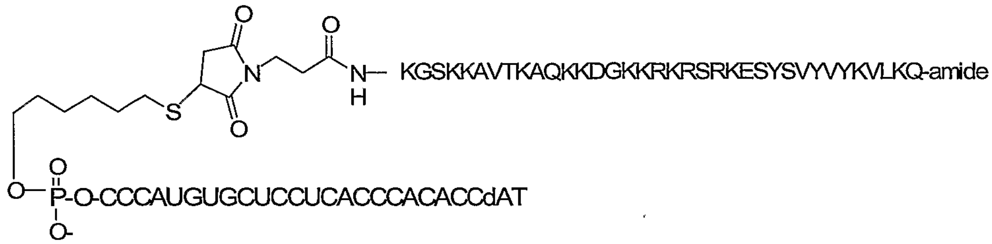
Example conjugate preparation 1: Conjugation Between Peptide PN277 (H2B 13-48) (SEQ ID NO: 37) and 5'of Antisense Strand CN950asen (N163asen) (SEQ ID NO: 60) having the following structure:



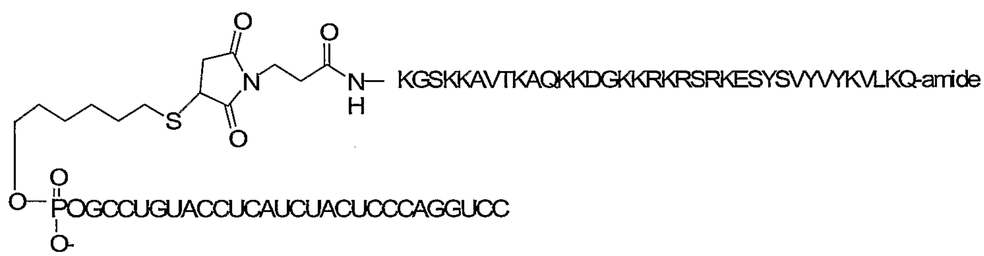
Oligo CN950asen was dissolved in 0.5 mL of water to which was added 2 mL of buffer A (20 mM Tris-HCl, pH 6.8, 50% formamide). Peptide (PN0277) was subsequently added which resulted in the formation of a precipitate that was solubilized by the additional 1 mL of 2 M TEAA buffer. Upon completion of the reaction, the solvent was removed under reduced pressure and the resulting solid was dissolved in buffer A (20 mM Tris-HCl, pH 6.8, 50% formamide). The material was loaded onto an Amersham Resource Q column and washed with 5 column volumes of buffer A at 6 mL/min. Separation was accomplished by running a linear gradient of Buffer B (20 mM Tris-HCl, pH 6.8, 50% formamide, 1 M NaCl) from 15-60% for 15 column volumes at a flow rate of 6 mL/min. The purified conjugate was desalted by slidalyzer dialysis cassettes (3.5 K MWCO) against PBS. The amount of the conjugate was determined spectrophotometrically based on the calculated molar absorption coefficient at λ=260 nm. Purity of the conjugate was confirmed by RP-HPLC. The peptide conjugate antisense strand and complimentary antisense strand were annealed in 50 mM potassium acetate, 1 mM magnesium acetate and 15 mM HEPES pH 7.4 by heating at 90°C for 2 min followed by incubation at 37°C for 1 h. The formation of the double stranded RNA conjugate was confirmed by non denaturing (15%) polyacrylamide gel electrophoresis and staining with ethidium bromide.

Example conjugate preparation 2: The following conjugates were synthesized using the methods and procedures described above in Example conjugate preparation 1.

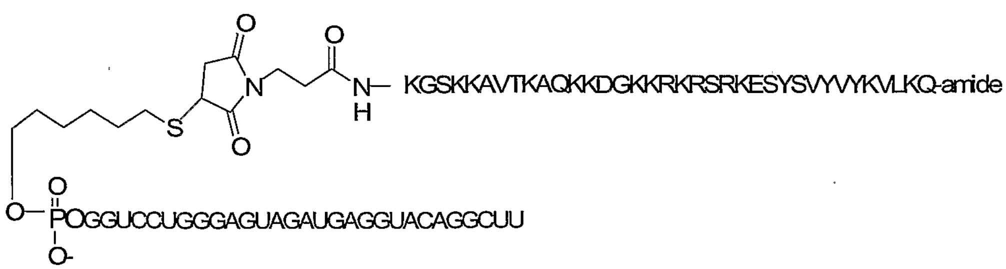
A conjugate between peptide PN277 (SEQ ID NO: 37) and oligo CN952sen (N163sen) (SEQ ID NO: 59) having the following structure:



A dicer substrate 27 mer conjugate between peptide PN277 and oligo CN740sen (SEQ ID NO: 63) having the following structure:



A dicer substrate 29 mer conjugate between peptide PN277 and oligo CN741asen (SEQ ID NO: 64) having the following structure:



Cells and Cell Culture

Mouse tail fibroblast cells were derived from the tails of C57BL/6J mice or tg197 hTNF-α transgenic mice. Tails were removed and cut into small sections with a razor blade. The sections were washed three times with PBS then incubated in a shaker at 37°C with 0.5 mg/ml collagenase, 100 units/ml penicillin and 100 ug/ml streptomycin to disrupt tissue. Tail sections were then cultured in DMEM media with supplements (as described above). 9L/lacZ cells are rat gliosarcoma fibroblast cells, constitutively expressing LacZ, obtained from ATCC (#CRL-2200) were also grown in DMEM media with supplements (as described above).

Isolated human monocytes were maintained in Iscove's modified Dulbecco's medium (IMDM) with 4 mM L-glutamine, nonessential amino acids and 10% fetal bovine serum. Mouse tail fibroblasts (MTF) were maintained in Dulbecco's Modified Essential Medium (DMEM) with a supplement of 1 mM sodium pyruvate, nonessential amino acids, and 20% fetal bovine serum. All cells were cultured at 37°C and 5% CO2 supplemented with an antibiotic mixture containing 100 units/ml penicillin, 100 ug/ml streptomycin and 0.25 mg/ml Fungizone (Invitrogen, Carlsbad, CA).

Human Monocyte Isolation and Purity

Fresh human blood samples from healthy donors were purchased from Golden West Biologicals (Temecula, CA). For isolation of monocytes, blood samples were diluted with PBS at a 1:1 ratio immediately after receiving. Peripheral blood mononuclear cells (PBMC) were first isolated by Ficoll (Amersham, Piscataway, NJ) gradient from whole blood. Monocytes were further purified from PBMCs using Miltenyi CD14 positive selection kit (MILTENYI BIOTEC GmbH, Germany) following the manufacturer's instructions. The purity of the monocyte preparation was greater than 95% as judged by flow cytometry after staining cells with anti-CD14 antibody (BD Biosciences, San Jose, CA). Purified human monocytes were maintained overnight in complete media (described above) before induction and knockdown assays.

Human Monocyte Activation

Activation of human monocytes was performed by adding 0.1-1.0 ng/ml of Liposaccharides, LPS (Sigma, St Louis, MO) to the cell culture to stimulate tumor necrosis factor-α (TNF-α) production. Cells were harvested 3 hours after incubation with LPS and mRNA levels were determined by Quantigene assay (Genospectra, Fremnont, CA) according to the manufacturer's instructions. Post-induction changes in TNF-α levels were determined by ELISA (BD Biosciences, San Jose, CA), following the manufacturer's protocol.

Human TNF-α Knockdown Assays for mRNA (bDNA) and Protein (ELISA)

For TNF-α knockdown experiments, monocytes were seeded in a 96 well plate with OptiMEM (Invitrogen, Carlsbad, USA) at 100K cells/100ul/well. Peptide and siRNA were complexed in OptiMEM for 5 minutes and then fetal bovine serum (FBS) was added to the complex to make 3% FBS final concentration. For cells transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. All transfections were carried out by incubating cells at 37°C and 5% CO_2 for 3 hours, followed by removing the transfection reagent, replenishing cells with complete media and culturing overnight. bDNA assays (Quantigene assay from Genospectra, Fremont, CA) were performed following the manufacturer's instructions. For the ELISA assay, samples from cell culture media were used directly. For the analysis of experiments involving transgenic mice, plasma samples were separated from whole blood collected by orbital bleeding. Plasma levels of hTNF-α were determined by ELISA at a 1:2 dilution according to the manufacturer's instructions (R&D systems, Minneapolis, MN).

Flow Cytometry

Flow cytometry analysis was performed using Beckman Coulter FC500 cell analyzer (Fullerton, CA). The instrument was adjusted according to the fluorescence probes used (FAM or Cy5 for siRNA and FITC or PE for CD14). Propidium iodide (Fluka, St Louis, MO) and AnnexinV (BD Bioscience, San Jose, CA) were used as indicators of cell viability and cytotoxicity.

Dicer Mediated siRNA Degradation Assay

siRNA and siRNA/polypeptide conjugates were incubated with Dicer endonuclease (Stratagene Cat. # 240100) to determine whether they were susceptible to degradation. The manufacturer's protocol was followed. Briefly, the digestion reaction was performed in a total volume of 10 μL and allowed to incubate overnight at 37^0C. Following the overnight incubation, a 2 μL sample of the digestion mixture was mixed with 2X loading dye and analyzed by gel electrophoresis on a 15% TBE with Urea and a 15% TBE non-denaturing polyacrylamide gel.

Monitoring Dicer Degradation of RNAs by LC-MS

LC-MS was run using an XTerra C18 column, 2.5 μm, 2.1x50 mm (Waters, Corp.) held at 65°C. The mobile phase was 100 mM hexafluoroisopropanol, 7 mM triethylamine, and elution with 100% Methanol. The gradient was 5-16% over 40 min. The eluent was split into a PDA and a Waters Micromass ZQ ESI single-quad mass spectrometer run in negative ion mode.

The capillary voltage was 3.0kV and the cone voltage was 45V. Desolvation took place at 300°C, assisted with 600 L/hr N_2. The source was held at 90°C. The acquisition scan rate was 1000-2000 m/z over 1 sec.

Example 2

Dicer Endonuclease Substrate siRNAs Screened for hTNF-α Knockdown Activity

The present example illustrates the exemplary siRNAs of the present invention that were screened for effective reduction of hTNF-α gene expression levels. The significance of targeting the hTNF-α gene is that it is implicated in mediating the occurrence or progression of rheumatoid arthritis (RA) when over-expressed in human and other mammalian subjects. Therefore, targeted reduction of hTNF-α gene expression can be used as a treatment for RA.

Recent evidence suggest that RNA duplexes 25-30 nucleotides in length can be up to 100-fold more effective in reducing the expression levels of a target gene than corresponding shorter RNA duplexes. This enhanced knockdown activity is attributed to the fact that these longer siRNA duplexes are a substrate for the dicer endonuclease (RNAse III). Table 3 below shows siRNA duplexes ranging in length from 21 to 27 nucleotides. YC12 and N161-N164 were screened for sequence-specific post-transcriptional gene silencing of the hTNF-α gene. This initial screening process involved transfecting the individual siRNAs listed in Table 3 with Lipofectamine 2000 (Invitrogen, Carlsbad, Ca) into LPS stimulated human monocytes.

Table 3

siRNAs Targeted Against hTNF-α

siRNA	Nucleic Acid Sequence
YC12	SEQ ID NO 53 5'-GCCUGUACCUCAUCUACUCUU-3' SEQ ID NO 54 3'-UUCGGACAUGGAGUAGAUGAG-5'
N161	SEQ ID NO 55 5'-GCCUCUUCUCCUUCCUGAUCGUGdGdC-3' SEQ ID NO 56 3'-GUCGGAGAAGAGGAAGGACUAGCACCG-5'
N162	SEQ ID NO 57 5'-GCCUGCUGCACUUUGGAGUGAUCdGdG-3' SEQ ID NO 58 3'-GACGGACGACGUGAAACCUCACUAGCC-5'
N163	SEQ ID NO 59 5'-CCCAUGUGCUCCUCACCCACACCdAT-3' SEQ ID NO 60 3'-GUGGGUACACGAGGAGUGGGUGUGGUA-5'
N164	SEQ ID NO 61 5'-ACCUCAUCUACUCCCAGGUCCUCdTdT-3' SEQ ID NO 62 3'-CAUGGAGUAGAUGAGGGUCCAGGAGAA-5'
CN740 sen(5'-3')	SEQ ID NO 63 5'-GCCUGUACCUCAUCUACUCCCAGGUCC-3'
CN741 asen(5'-3')	SEQ ID NO 64 5'-GGUCCUGGGAGUAGAUGAGGUACAGGCUU-3'

Sequences YC12 and N161-N164 are listed in Table 3 as 5'-3' sense (top) and 3'-5' antisense (bottom).

Each of YC12 and N161-N164 were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, Ca) at a 0.16 nM, 0.8 nM, 4 nM and 20 nM concentration. Table 4 summarizes the TNF-α knockdown activity for each siRNA expressed as a percentage. Qneg represents a random siRNA sequence and functioned as the negative control. The observed Qneg knockdown activity is normalized to 100% (100% gene expression levels) and the knockdown activity for each siRNA was presented as a relative percentage of the negative control. The data in Table 4 shows that the siRNAs N161, N162 and N164 did not have a significant effect on TNF-α mRNA levels when compared to the Qneg siRNA negative control. In contrast, the siRNA Y12 reduced TNF-α mRNA levels to 66% of the Qneg negative control while the siRNA N163 reduced mRNA levels to 57% of the Qneg negative control.

The data in Table 4 shows that the dicer substrate siRNAs N163 and YC12 effectively reduced hTNF-α mRNA levels in human monocytes. The siRNA N163 was chosen for further characterization.

Table 4

TNF-α Knockdown Activity of Lipofectamine Transfected siRNA

siRNA	siRNA Concentration	% TNF-α mRNA Expression Level
Qneg (Negative Control)	0.16 nM	100%
	0.8 nM	
	4 nM	
	20 nM	
YC12	0.16 nM	70%
	0.8 nM	83%
	4 nM	66%
	20 nM	82%
N161	0.16 nM	100%
	0.8 nM	99%
	4 nM	110%
	20 nM	123%
N162	0.16 nM	89%
	0.8 nM	81%
	4 nM	91%
	20 nM	112%
N163	0.16 nM	76%
	0.8 nM	66%
	4 nM	65%
	20 nM	57%
N164	0.16 nM	86%
	0.8 nM	83%
	4 nM	71%
	20 nM	76%

Example 3

siRNA/Polypeptide Conjugates Effectively

Reduced hTNF-α mRNA levels in Human Monocytes

The present example demonstrates that the exemplary dicer substrate siRNA of the present invention effectively reduced the expression of hTNF-α mRNA levels when conjugated to the exemplary polynucleotide delivery-enhancing polypeptide of the invention. A summary of the siRNA/polypeptide conjugate knockdown activity is shown below in Table 5.

The data presented in Table 4 of Example 2 showed that the siRNA N163 effectively reduced hTNF-α mRNA levels when transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, Ca) and thus is an excellent candidate for the treatment and/or prevention of one or more TNF-α-associated inflammatory condition(s). However, cationic lipids can be cytotoxic and, therefore, not appropriate for *in vivo* delivery applications in the treatment of disease. Thus, to overcome this deficiency in cationic lipid mediated delivery, the exemplary N163 siRNA of the present invention was conjugated to a polynucleotide delivery-enhancing polypeptide, which has minimal to no cytotoxic effect when used as a delivery vehicle. Conjugation of the delivery polypeptide to the dicer substrate form (27-mer) of the siRNA sequence allows the polypeptide to be removed from the siRNA prior to RISC loading, thus reducing the possibility that the polypeptide will inhibit RISC activity. In effect, these conjugates efficiently deliver a precursor siRNA into the cytoplasm that once processed mediate effective knockdown of the desired target gene.

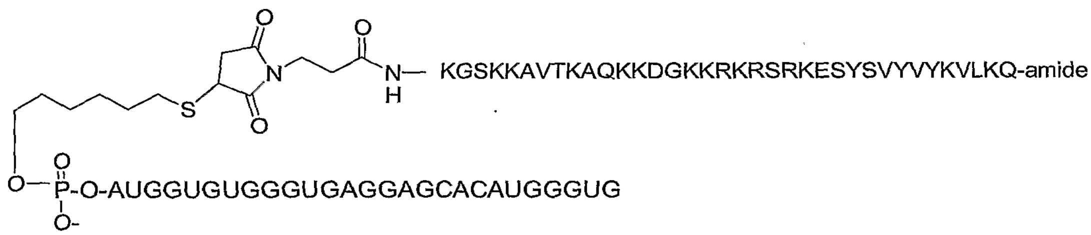
The exemplary polynucleotide delivery-enhancing polypeptide (PN277) of the present invention was derived from the amino acid sequence of the human histone 2B (H2B) protein and its primary structure is as follows:

PN277

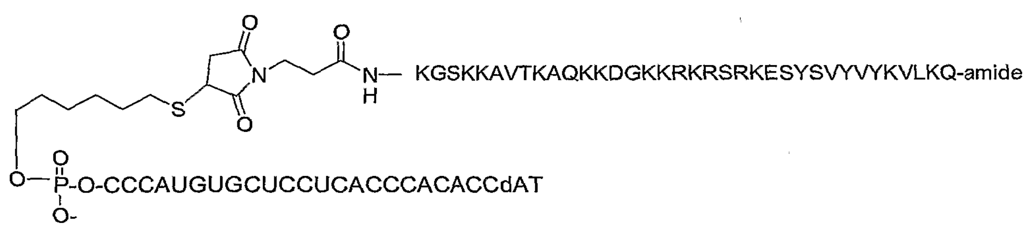
NH2-KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ-amide (SEQ ID NO: 37)

Conjugates were generated by covalently linking the exemplary polynucleotide delivery-enhancing polypeptide PN277 of the present invention to the 5'-end of the sense strand or the 5'-end of the anti-sense strand of exemplary N163 siRNA of the present invention. Examples of the siRNA/polypeptide conjugates are as follows:

The polypeptide PN277 conjugated to the 5'-end of the anti-sense strand of the N163 siRNA (dsCoP277nfR950; SEQ ID NOs: 37 and 35). The sense strand of the N163 siRNA is not shown.



The polypeptide PN277 conjugated to the 5'-end of the sense strand of the N163 siRNA (dsCoP277nfR952; SEQ ID NOs: 37 and 36. The anti-sense strand of N163 siRNA is not shown.



The polypeptide PN277 conjugated to the 5'-end of the sense strand of the Qneg siRNA used herein as a negative control is not shown.

In the present example, each siRNA/polypeptide conjugate described above, including the Qneg siRNA/polypeptide conjugate, was incubated with human monocytes at a 1 nM, 10 nM, 100 nM and 200 nM concentration. Table 5 summarizes the TNF-α knockdown activity for each siRNA/polypeptide conjugate expressed as a percentage. The observed Qneg knockdown activity is normalized to 100% (100% gene expression levels) and the knockdown activity for each siRNA was presented as a relative percentage of the negative control.

The data in Table 5 shows that the N163 siRNA duplex conjugated to the polynucleotide delivery-enhancing polypeptide effectively reduced hTNF-α mRNA expression levels regardless whether the polypeptide was covalently linked to the 5'-end of the sense strand or the 5'-end of the anti-sense strand of the N163 siRNA molecule. More specifically, the siRNA/polypeptide conjugate dsCoP277nfR952 reduced hTNF-α mRNA levels to 62% of the Qneg/polypeptide conjugate negative control mRNA levels while the dsCoP277nfR950 reduced hTNF-α mRNA levels to 38% of the Qneg/polypeptide conjugate negative control mRNA levels.

Table 5

siRNA Dicer Substrate Polypeptide Conjugate TNF-α Knockdown Activity

siRNA/Polypeptide Conjugate	siRNA/Polypeptide Conjugate Concentration	% TNF-α mRNA Expression Level
Qneg/PN277 (negative control)	1 nM	100%
	10 nM	
	100 nM	
	200 nM	
dsCoP277nfR950	1 nM	120%
	10 nM	95%
	100 nM	73%
	200 nM	38%
dsCoP277nfR952	1 nM	80%
	10 nM	78%
	100 nM	62%
	200 nM	68%

The data presented in Table 5 show the surprising and unexpected discovery that the exemplary dicer substrate siRNA of the present invention effectively reduced the expression of hTNF-α mRNA levels when conjugated to the exemplary polynucleotide delivery-enhancing polypeptide of the invention. Further, the conjugate knockdown activity exceeded the knockdown activity exhibited by the same siRNA delivered via lipofectamine.

Example 4

siRNA Conjugated to a Polynucleotide

Delivery-Enhancing Polypeptide is Processed by Dicer Enodnuclease

This example demonstrates that siRNAs conjugated to a polynucleotide delivery-enhancing polypeptide are degraded by the dicer endonuclease (RNase III). In the instant example, the three siRNA/polypeptide conjugates (Qneg, dsCoP277nfR950 and dsCoP277nfR952) shown in Example 3 were incubated in the presence or absence of dicer endonuclease. In addition, the N163 siRNA duplex without the polynucleotide delivery-enhancing polypeptide was incubated in the presence or absence of the Dicer endonuclease. The purpose was to determine whether the siRNA was a target for dicer mediated degradation and whether the polypeptide was removed from the covalently linked siRNA duplex, which is essential to prevent polypeptide mediated inhibition of siRNA RISC loading. Inhibition of the RISC complex would prevent post-transcriptional gene silencing of the targeted gene.

A comparison by polyacrylamide gel electrophoresis is shown in Figure 1. In Figure 1, both the non-dicer incubated and dicer incubated Qneg siRNA/polypeptide conjugate migrated as "sharp" bands of the same molecular weight, which, as expected, indicated that the Qneg siRNA/polypeptide conjugate did not degrade in the presence or absence of the dicer endonuclease. The exemplary N163 siRNA (no polypeptide) of the present invention in the absence of the dicer endonuclease migrated as a "sharp" band on the polyacrylamid gel. However, a slightly shorter N163 siRNA duplex was observed when it was incubated with dicer as evidenced by a slightly smaller molecular weight band compared to the non-incubated N163 siRNA. Differential degradation was observed between the exemplary N163 siRNA covalently linked to the polynucleotide delivery-enhancing polypeptide by the 5'-end of the anti-sense strand (dsCoP277nfR950) and the exemplary N163 siRNA covalently linked to the polynucleotide delivery-enhancing polypeptide by the 5'-end of the sense strand (dsCoP277nfR952). Both these siRNA/polypeptide conjugates in the absence of dicer migrated as distinct bands of equivalent molecular weight indicating no degradation, as expected. However, in the presence of the dicer endonuclease, dsCoP277nfR952 (sense strand linkage) two different molecular weights bands were observed. The molecular weight of one band was equivalent to the non-degraded dsCoP277nfR952 and the second band had a molecular weight equivalent to the dicer degraded N163 siRNA (no polypeptide), which suggested that the covalently linked N163 siRNA separated from the polypeptide. The intensity of the higher molecular weight band (i.e., the non-degraded N163 siRNA/polypeptide) was approximately 2-fold more intense than the lower molecular weight band (i.e., degraded N163

siRNA/polypeptide), which indicated that a majority of dsCoP277nfR952 (sense strand linkage) was not degraded. In contrast, dsCoP277nfR950 (anti-sense strand linkage) exhibited greater susceptibility to dicer mediated degradation as evidenced by a relatively faint band equivalent in size to the non-degraded dsCoP277nfR950 and a more intense molecular weight band equivalent in size to the dicer degraded N163 siRNA (no polypeptide). The relatively higher level of susceptibility to dicer mediated degradation for the siRNA/polypeptide dsCoP277nfR950 (anti-sense strand linkage) compared to dsCoP277nfR952 (sense strand linkage) correlates strongly with the greater knockdown activity observed for dsCoP277nfR950 (anti-sense strand linkage; refer to Example 2). As greater siRNA/polypeptide dicer susceptibility indicates a reduced possibility of polypeptide mediated interference of the RISC complex and thus greater silencing of the targeted gene.

These data indicate that the exemplary siRNA conjugated to the polynucleotide delivery-enhancing polypeptide of the present invention is susceptible to dicer mediated degradation and represents an ideal candidate for the effective delivery of therapeutic pre-cursor siRNAs into cells for purposes of targeted gene silencing.

Digestion of siRNA N163 by dicer endonuclease results in 21-mer RNAs. Figure 2 shows the RP-HPLC analysis of dicer endonuclease processing kinetics for non-conjugated siRNA N163 duplex. In Figure 2(A) is shown the RP-HPLC for unprocessed N163 duplex. In Figures 2(B-E) is shown the RP-HPLC for N163 duplex incubated with dicer endonuclease for (B) 1 hr, (C) 2.5 hr, (D) 5 hr, and (E) 7 hr. These data are shown in the chart in Figure 3.

The identity of RNAs after 7 hours digestion of siRNA N163 by dicer endonuclease as shown in Figure 2(E) was confirmed by ESI-MS analysis as shown in Figure 4. Figure 4 shows peaks at mass 6606.6 corresponding to the 21-mer sense strand dicer cleavage product of N163, and mass 6965.7 corresponding to the 21-mer antisense strand dicer cleavage product of N163.

The identity of RNAs after digestion of conjugated siRNA N163 by dicer endonuclease was confirmed by ESI-MS analysis as shown in Figure 5. In Figure 5 is shown the ESI-MS of dicer endonuclease processing for a conjugated siRNA having polypeptide PN857 conjugated to siRNA N163. The exemplary polynucleotide delivery-enhancing polypeptide PN857 of this invention was derived from the amino acid sequence of the human histone 2B (H2B) protein and its primary structure is as follows:

PN857

Mal-KGSKKAVTKAQKKEGKKRKRSRKESYSVYVYKVLKQ-amide

(SEQ ID NO: 52)

The polypeptide PN857 was conjugated to the 5'-end of the anti-sense strand of N163 siRNA.

In Figure 5(A), the control incubation of the conjugate duplex without dicer endonuclease present was obtained. Figure 5(A) shows peaks at mass 13436.2 corresponding to the conjugate of 27-nt antisense strand of N163 with polypeptide PN857, and at mass 7835.3 corresponding to the 25-nt sense strand of N163.

In Figure 5(B), incubation of the conjugate duplex with dicer endonuclease present for 8 hours was obtained. Figure 5(B) shows peaks at mass 13436.1, corresponding to the 27-nt antisense strand conjugate of N163 with polypeptide PN857, at mass 7835.6, corresponding to the 25-nt sense strand of N163, at mass 6966.3, corresponding to the 21-mer antisense strand dicer cleavage product of the conjugated 27-mer of N163, and at mass 6607.6, corresponding to the 21-mer sense strand dicer cleavage product of the 25-mer of N163.

Example 5

Dicer Substrate siRNA/Polypeptide Conjugates do not Elicit an Interferon Response

The present example demonstrates that the exemplary siRNA/polypeptide conjugates of the instant invention do not elicit an interferon response when incubated with human monocytes. Interferon responsiveness is a potential side-effect of transfecting cells with siRNAs. Thus, a study was performed *in vitro* to assess whether siRNA/polypeptide dsCoP277nfR950 (anti-sense strand linkage) and dsCoP277nfR952 (sense strand linkage) elicit an interferon response. The Qneg siRNA/polypeptide conjugate (CoP840) served as a negative control while interferon-1 (IFN-1) was used as a positive control. Each conjugate was tested at 1 nM, 10 nM, 100 nM and 200 nM concentration. The molecular marker MIP1α was used to assay for interferon responsiveness. As expected, the Qneg siRNA/polypeptide conjugate did not induce MIP1α expression and the positive control IFN-1 induced a significant amount of MIP1α expression. The conjugates dsCoP277nfR950 and dsCoP277nfR952 induced MIP1α levels comparable to that of the Qneg siRNA/polypeptide negative control, indicating that these conjugates do not elicit interferon responsiveness at concentrations where they exhibit potent knockdown activity of TNF-α (refer to Table 5, Example 3).

Example 6

Vero Cell Transfection in 24 Well Plate

This Example discloses a method for introducing siRNA into a target cell.

Vero cells are grown in DMEM medium containing 10% FBS, 2 mL L-glutamine, 10 nM Hepes, 100 units/ml pen/strep. One day before transfection, log-phase cultures of Vero cells are seeded in 24 well plate at 50,000 cells/well. Before transfection, make up an appropriate stock solution of L2K (Lipofectamine™ 2000; Invitrogen) for all siRNA doses. Mix L2K gently in OptiMEM® cell culture medium (Invitrogen) and add to diluted siRNA. Incubate the mixture for 15-30 minutes at room temperature to form the transfection complex. At the end of the incubation, culture supernatant is aspirated and 150 µl DMEM medium containing 10% FBS is added to each well. Next 150 µl of each transfection complex is added to each well in triplicate. Rock plate back and forth gently to mix. After incubation for 3 hours at 37°C, the supernatant is removed and 1 ml complete media is added per well. For Cy5-siRNA detection, incubate cells overnight and examine the florescence under microscopy. On the next day, stain cells with Hoescht DNA stain by added 1.5 µl of Hoescht stock per well for toxicity detection. Swirl wells gently and incubate for 15 minutes at 37°C. Cells can be examined immediately using the fluorescent microscope using Cy5 and UV filters. Examine cells using phase contrast for toxicity of transfection compounds.

Example 7

Hemagglutination Assay (HA Assay)

This Example discloses a hemagglutination assay that may be used to calculate the titer of viruses produced from virus-infected cells.

Hemagglutination assays (HA) are used to quantify viruses in cell culture supernatants. Some viral families, e.g., influenza virus, have surface or envelope proteins, that are able to agglutinate (stick to) human or animal red blood cells (RBC) and bind to the N-acetylneuraminic acid on the RBC surface. Because each virus has many cell surface proteins, one virus can stick to more than one RBC. The result of this is that the virus-bound RBCs become crosslinked by the viruses, leading to the formation of a type of RBC "lattice." Once the lattice is formed, the agglutinated RBCs can be counted and the virus titer calculated. A reduction in virus titer in the siRNA-transfected cells relative to the virus titer in cells that have not been transfected with siRNA indicates that the siRNA molecules have interfered with the expression of the targeted viral gene or genes.

Prepare 0.5% chicken red blood cells in PBS, every 20 ml for 4 plates. Transfer 100 μl of culture supernatant from each sample to each well of the first row of the 96-well plate, v-bottom. Change tips between samples. Perform in the tissue culture hood if sterile is required. Use multi-channel pipette to add 50 μl PBS per well to the rest of the plates, except the first row that received viral supernatant. Use multi-channel pipette to aspirate 50 μl culture supernatant from the first row and mix with the 50 μl PBS in the second row. Pipette up and down 3 times. Use multi-channel pipette to aspirate 50 μl diluted sample from the second row and mix with the 50 μl PBS in the third row. Pipette up and down 3 times. Repeat 4 and 5 until reaching the last row. Discard 50 μl diluted sample. Add 50 μl 0.5% chicken red blood cells into each well. Incubate the plates on ice for 1 hour. Read the HA results and calculate the virus titer.

Example 8

24-well Vero Cell Transfection/Infection Titer Knock-down Screening Assay

This Example discloses an assay that can be used to determine whether gapped or nicked duplex siRNA of the present invention are capable downregulating expression of one or more viral target genes.

In this Example, mammalian cells are transfected with gapped or nicked duplex siRNA homologous to viral RNA. After transfection, the transfected cells are infected with a virus that contains one or more genes with sequences homologous to the transfected siRNA molecules according to this invention.

Vero cells are transfected with gapped or nicked duplex siRNA according to the method of Example 6. Towards the end of incubation of the siRNA and the Vero cells, virus dilutions are made up for infection step. The virus samples are diluted so as to achieve a desired multiplicity of infection (MOI). The virus samples are diluted in PBS/BSA/PS, and an appropriate amount of virus solution is added to each well. After incubating for 1 hour at 37°C, an amount of infection media (DMEM medium containing 0.3% BSA, 2 mL L-glutamine, 10 nM Hepes, and 100 units/ml pen/strep, plus trypsin) is added to each well. Incubate for 48 hrs. at 37°C. At the end of incubation, the supernatant is harvested and stored in 4°C. siRNA-directed viral RNA interference is measured by running the HA assay according to Example 7.

Example 9

Influenza-Specific Dicer Endonuclease

Substrate siRNAs Screened for Knockdown Activity

This example illustrates the exemplary influenza-specific siRNAs presented herein that were screened for effective reduction of influenza viral titer in infected Vero cells. The significance of a reduction in viral titer in the influenza-specific siRNA-transfected cells relative to the viral titer in cells transfected with non-targeting control siRNA is that it indicates that the influenza-specific siRNA molecules have interfered with the expression of the targeted viral gene or genes.

Table 6 below shows siRNA duplexes specific for influenza, which are listed as 5' – 3' sense (top) and 5' – 3' antisense (bottom).

Table 6

Influenza-Specific Dicer Substrate siRNAs

27 mer DX #	25 mer counterpart	siRNA Sequence
DX2852	G3817 (DX2825)	SEQ ID NO: 65 sense 5'-AGACAGCGACCAAAAGAAUUCGGdAdU-3' SEQ ID NO: 66 antisense 5'-AUCCGAAUUCUUUUGGUCGCUGUCUdTdT-3'
DX2855	G6124 (DX2820)	SEQ ID NO: 67 sense 5'-AUGAAGAUCUGUUCCACCAUUGAdAdG-3' SEQ ID NO: 68 antisense 5'-CUUCAAUGGUGGAACAGAUCUUCAUdTdT-3'
DX2858	G6129 (DX2819)	SEQ ID NO: 69 sense 5'-GAUCUGUUCCACCAUUGAAGAACdUdC-3' SEQ ID NO: 70 antisense 5'-GAGUUCUUCAAUGGUGGAACAGAUCdTdT-3'
DX2861	G8286 (DX2822)	SEQ ID NO: 71 sense 5'-UUGAGGAGUGCCUGAUUAAUGAUdCdC-3' SEQ ID NO: 72 antisense 5'-GGAUCAUUAAUCAGGCACUCCUCAAdTdT-3'
DX2956	G1498 (DX2744)	SEQ ID NO: 73 sense 5'-GGAUCUUAUUUCUUCGGAGACAAdTdG-3' SEQ ID NO: 74 antisense 5'-CAUUGUCUCCGAAGAAAUAAGAUCCdTdT-3'

Vero cells were seeded at 1.5 x 10^4 cells per well in a 96 well plate the day before transfection in 100 µl 10% FBS/DMEM media per well. 100, 20, or 5 nM of each influenza specific siRNA or non-targeting control siRNA, Qneg, was complexed with 0.3 µl (1 mg/mL stock) of lipofectamine 2000 (Invitrogen) and incubated for 20 minutes at room temperature in 25 µl OptiMEM (total volume)(Gibco). Supernatant from each well of seeded Vero cells was

removed, and 75 μL 10% DMEM complete media was added. The 25 μl of the siRNA/lipofectamine complex in OptiMEM was then added to each well. Triplicate wells were tested for each condition. An additional control well with no transfection condition was prepared. Three hours post transfection, the media was removed. Each well was washed 1X with 100 μl 1X PBS containing 0.3% BSA/10 mM HEPES/PS. 30 μls of PR8 influenza virus at MOI = 0.1 was added to each well for infection. The plate was rocked for 1 hour at room temperature. 100 μl DMEM containing 0.3% BSA/10 mM HEPES/PS + 4 ug/ml trypsin was added to each well gently on the side of the well, since the cells start to detach following infection. The plate was incubated at 37°C, 5% CO2, for 48 hours. 50 μl supernatant from each well was tested in duplicate by HA Assay.

Each of DX2852, DX2855, DX2858, DX2861, DX2956 and G1498 influenza-specific dicer substrate siRNAs and Qneg, a non-targeting control siRNA, at 5 nM, 20 nM, and 100 nM concentrations, were complexed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and used to transfect seeded Vero cells. Following transfection, the cells were infected with PR8 influenza virus at MOI (multiplicity of infection) of 0.1. To quantify the virus produced, supernatant from the wells containing the infected cells was tested by hemagglutinin (HA) assay as described in Example 7. Table 7 summarizes the knockdown of influenza gene expression activity for each siRNA, expressed as the percentage of viral titer reduction (1 – (HA units with influenza siRNA treatment / HA units with control siRNA treatment).

Table 7

Percentage Viral Titer Reduction in

siRNA-Transfected Vero Cells Infected with Influenza Virus

siRNA Conc.	DX2852	DX2855	DX2858	DX2861	DX2956	G1498
100 nM	0	25	0	15	50	50
20 nM	25	15	40	0	50	50
5 nM	40	25	0	40	50	50

The data in Table 7 show that influenza-specific dicer substrate siRNA DX2956 effectively reduced influenza titers in Vero cells infected with influenza virus. DX2956 showed similar activity as G1498, while the other four dicer substrate siRNAs exhibited less activity than DX2956. These data demonstrated that the siRNA DX2956 effectively reduced influenza mRNA levels when transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, Ca) and thus was an excellent candidate for the treatment and/or prevention of influenza infection.

Although the foregoing invention has been described in detail by way of example for purposes of clarity of understanding, it will be apparent to the artisan that certain changes and modifications may be practiced within the scope of the appended claims which are presented by way of illustration not limitation. In this context, various publications and other references have been cited within the foregoing disclosure for economy of description. Each of these references is incorporated herein by reference in its entirety for all purposes. It is noted, however, that the various publications discussed herein are incorporated solely for their disclosure prior to the filing date of the present application, and the inventors reserve the right to antedate such disclosure by virtue of prior invention.

WHAT IS CLAIMED IS:

1. A composition comprising:

(a) a double stranded ribonucleic acid (dsRNA) molecule, wherein the strands have lengths from about 25 to about 30 base pairs which may be the same or different; and

(b) a peptide comprising about 5 to about 40 amino acids, wherein the peptide contains the amino acid sequence KVLKQ (SEQ ID NO: 51);

wherein the dsRNA molecule is conjugated to the peptide.

2. The composition of claim 1, wherein the dsRNA is an siRNA.

3. The composition of claim 2, wherein the siRNA contains a sequence homologous to a portion of the sequence of a human TNF-alpha gene.

4. The composition of claim 2, wherein the siRNA contains a sequence homologous to a portion of the sequence of a viral gene.

5. The composition of claim 4, wherein the source of the viral gene is an influenza virus.

6. The composition of claim 1, further comprising a carrier.

7. The composition of claim 1, wherein the dsRNA has a 2 bp 3' antisense strand overhang.

8. The composition of claim 1, wherein the dsRNA has a 2 bp 3' sense strand overhang.

9. The composition of claim 1, wherein the dsRNA has no overhang.

10. The composition of claim 1, wherein the strands have lengths from about 25 to about 29 base pairs which may be the same or different.

11. The composition of claim 1, wherein the dsRNA molecule consists of a sense RNA strand and an antisense RNA strand, and the peptide is conjugated to the 5' end of the antisense strand.

12. The composition of claim 1, wherein the amino acid sequence of the peptide is selected from the group consisting of:
KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 33);
KKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 42);
VTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 43);
AQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 44);
KDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 45);
KKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 46);
KRSRKESYSVYVYKVLKQ (SEQ ID NO: 47);
RKESYSVYVYKVLKQ (SEQ ID NO: 41); SYSVYVYKVLKQ (SEQ ID NO: 48);
VYVYKVLKQ (SEQ ID NO: 49); YKVLKQ (SEQ ID NO: 50); and
KVLKQ (SEQ ID NO: 51).

13. The composition of claim 1, wherein the peptide is conjugated to a molecule that binds to a cell in an animal.

14. A method for ameliorating inflammation associated with TNF-α comprising administering an ameliorating amount of a composition of any one of claims 1-13 to an animal.

15. The method of claim 14, wherein the inflammation occurs in arthritis.

16. The method of claim 14, wherein the inflammation occurs in psoriasis.

17. A method for inhibiting expression of a gene in an animal for ameliorating inflammation comprising administering a composition of a double stranded ribonucleic acid (dsRNA) molecule to said animal, wherein the pharmaceutical compositions comprises said dsRNA molecule and a peptide, wherein the dsRNA molecule comprises about 25 to about 30 base pairs, wherein said peptide comprises about 5 to about 40 amino acids and comprises an amino acid sequence KVLKQ (SEQ ID NO: 51), and wherein said dsRNA molecule is conjugated to said peptide.

18. The method of claim 17, wherein the inflammation occurs in arthritis.

19. The method of claim 17, wherein the inflammation occurs in psoriasis.

20. A method for ameliorating infection associated with influenza virus comprising administering an ameliorating amount of a composition of claim 1 to an animal.

21. A composition comprising:

(a) a small inhibitory nucleic acid (siRNA) molecule,

said siRNA molecule comprising a first RNA strand (A strand) of between about 15 nucleotides and about 50 nucleotides, a second RNA strand (B1 strand) of between about between about 1 nucleotide and about 25 nucleotides, and a third RNA strand (B2 strand) of between about 1 nucleotide and about 25 nucleotides;

wherein the dsRNA molecule is conjugated to the peptide.

wherein said B1 strand and said B2 strand are each complementary to non-overlapping regions of said A strand;

wherein a first double-stranded region (A:B1) is formed by annealing said B1 strand and said A strand; and

wherein a second double-stranded region (A:B2) is formed by annealing said B2 strand and said A strand; and

(b) a peptide comprising about 5 to about 40 amino acids,

wherein said siRNA molecule is conjugated to said peptide.

22. The composition of claim 21 wherein said A:B1 duplex is separated from said A:B2 duplex by a nick or by a gap wherein said gap results from at least one unpaired nucleotide in the A strand that is positioned between said A:B1 duplex and said A:B2 duplex.

23. The composition of claim 22 further comprising one or more unpaired nucleotide at the 3' end of either or both of said A strand, said B1 strand, and/or said B2 strand.

24. The composition of any of claims 22-23 wherein said A strand is between about 18 nucleotides and about 40 nucleotides.

25. The composition of claim 24 wherein said A strand is between about 20 nucleotides and about 32 nucleotides.

26. The composition of claim 25 wherein said A strand is 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or 31 nucleotides.

27. The composition of any one of claims 22-23 wherein said A:B1 duplex and said A:B2 duplex comprise, in sum, between about 15 base-pairs and about 40 base-pairs.

28. The composition of claim 27 wherein said A:B1 duplex and said A:B2 duplex comprise, in sum, between about 18 base-pairs and about 35 base-pairs.

29. The composition of claim 28 wherein said A:B1 duplex and said A:B2 duplex comprise, in sum, between about 20 base-pairs and about 30 base-pairs.

30. The composition of claim 29 wherein said A:B1 duplex and said A:B2 duplex comprise, in sum, either 21, 22, 23, 24, 25, 26, 27, 28, or 29 base-pairs.

31. The composition of claim 23 wherein said siRNA molecule comprises one or two single-strand 3' overhang(s) of between 1 nucleotide and 5 nucleotides.

32. The composition of any one of claims 22-23 wherein said A strand comprises the nucleotide sequence 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5' (SEQ ID NO: 76).

33. The composition of claim 22 wherein said B1 strand comprises the nucleotide sequence 5'GGAUCU3' (SEQ ID NO: 77).

34. The composition of claim 22 wherein said B2 strand comprises the nucleotide sequence 5'ACAAUG3' (SEQ ID NO: 90).

35. The composition of claim 22 wherein said A:B1 duplex is separated from said A:B2 duplex by a nick.

36. The composition of claim 35 wherein said B2 strand terminates with a 5' hydroxyl.

37. The composition of claim 35 wherein said B1 strand comprises the nucleotide sequence 5'GGAUCUUAUUU3' (SEQ ID NO: 135), wherein said B2 strand comprises the nucleotide sequence 5'CUUCGGAGTT3' (SEQ ID NO: 136), and wherein said A strand comprises the nucleotide sequence 5'CUCCGAAGAAAUAAGAUCCTT3' (SEQ ID NO: 137).

38. The composition of claim 35 wherein said B1 strand comprises the nucleotide sequence 5'GGATCTTATTT3' (SEQ ID NO: 144), wherein said B2 strand comprises the nucleotide sequence 5'CTTCGGAGTT3' (SEQ ID NO: 145), and wherein said A strand comprises the nucleotide sequence 5'CTCCGAAGAAATAAGATCCTT3' (SEQ ID NO: 146).

39. The composition of claim 35 wherein said B1 strand comprises the nucleotide sequence 5'CTCCGAAGAA3' (SEQ ID NO: 148), wherein said B2 strand comprises the nucleotide sequence 5'ATAAGATCCTT3' (SEQ ID NO: 149), and wherein said A strand comprises the nucleotide sequence 5'GGATCTTATT TCTTCGGAGTT3' (SEQ ID NO: 147).

40. The composition of claim 35 wherein said B1 strand comprises the nucleotide sequence 5'GGAUCUUAUUU3' (SEQ ID NO: 153), wherein said B2 strand comprises the nucleotide sequence 5'CUUCGGAGTT3' (SEQ ID NO: 154), and wherein said A strand comprises the nucleotide sequence 5'CUCCGAAGAAAUAAGAUCCTT3' (SEQ ID NO: 155).

41. The composition of claim 35 wherein said B1 strand comprises the nucleotide sequence 5'GGATCTTATTT3' (SEQ ID NO: 159), wherein said B2 strand comprises the nucleotide sequence 5'CTTCGGAGTT3' (SEQ ID NO: 160), and wherein said A strand comprises the nucleotide sequence 5'CTCCGAAGAAATAAGATCCTT3' (SEQ ID NO: 161).

42. The composition of claim 35 wherein said B1 strand comprises the nucleotide sequence 5'CTCCGAAGAA3' (SEQ ID NO: 166), wherein said B2 strand comprises the nucleotide sequence 5'ATAAGATCCTT3' (SEQ ID NO: 34), and wherein said A strand comprises the nucleotide sequence 5'GGATCTTATT TCTTCGGAGTT3' (SEQ ID NO: 165).

43. The composition of claim 35 wherein said B1 strand terminates with a 5' phosphate.

44. The composition of claim 43 wherein said B1 strand comprises the nucleotide sequence 5'GGAUCUUAUUU3' (SEQ ID NO: 138), wherein said B2 strand comprises the nucleotide sequence 5'CUUCGGAGTT3' (SEQ ID NO: 139), and wherein said A strand comprises the nucleotide sequence 5'CUCCGAAGAAAUAAGAUCCTT3' (SEQ ID NO: 140).

45. The composition of claim 43 wherein said B1 strand comprises the nucleotide sequence 5'GGATCTTATTT3' (SEQ ID NO: 141), wherein said B2 strand comprises the nucleotide sequence 5'CTTCGGAGTT3' (SEQ ID NO: 142), and wherein said A strand comprises the nucleotide sequence 5'CTCCGAAGAAATAAGATCCTT3' (SEQ ID NO: 143).

46. The composition of claim 43 wherein said B1 strand comprises the nucleotide sequence 5'CTCCGAAGAA3' (SEQ ID NO: 151), wherein said B2 strand comprises the nucleotide sequence 5'ATAAGATCCTT3' (SEQ ID NO: 152), and wherein said A strand comprises the nucleotide sequence 5' GGATCTTATTTCTTCGGAGTT 3' (SEQ ID NO: 150).

47. The composition of claim 43 wherein said B1 strand comprises the nucleotide sequence 5'GGAUCUUAUUU3' (SEQ ID NO: 156), wherein said B2 strand comprises the nucleotide sequence 5'CUUCGGAGTT3' (SEQ ID NO: 157), and wherein said A strand comprises the nucleotide sequence 5'CUCCGAAGAAAUAAGAUCCTT3' (SEQ ID NO: 158).

48. The composition of claim 43 wherein said B1 strand comprises the nucleotide sequence 5'GGATCTTATTT3' (SEQ ID NO: 162), wherein said B2 strand comprises the nucleotide sequence 5'CTTCGGAGTT3' (SEQ ID NO: 163), and wherein said A strand comprises the nucleotide sequence 5'CTCCGAAGAAATAAGATCCTT3' (SEQ ID NO: 164).

49. The composition of claim 43 wherein said B1 strand comprises the nucleotide sequence 5'CTCCGAAGAA3' (SEQ ID NO: 39), wherein said B2 strand comprises the nucleotide sequence 5'ATAAGATCCTT3' (SEQ ID NO: 40), and wherein said A strand comprises the nucleotide sequence 5'GGATCTTATTTCTTCGGAGTT3' (SEQ ID NO: 38).

50. A composition comprising:

(a) a small inhibitory nucleic acid (siRNA) molecule, said siRNA molecule comprising three strands A, B1, and B2 (A:B1B2);

wherein A:B1B2 comprises between about 14 total base-pairs and about 24 total base-pairs;

wherein A represents the sense strand and B1B2 represents the antisense strand;

wherein A is between about 19 nucleotides and about 25 nucleotides;

wherein B1 and B2 are each, individually, between about 1 nucleotide and about 15 nucleotides; and

wherein the combined length of B1+B2 is between about 13 nucleotides and about 23 nucleotides; and

(b) a peptide comprising about 5 to about 40 amino acids,

wherein said siRNA molecule is conjugated to said peptide.

51. A composition comprising:

(a) a small inhibitory nucleic acid (siRNA) molecule, said siRNA molecule comprising three strands A, B1, and B2 (A:B1B2);

wherein A:B1B2 comprises between about 16 total base-pairs and about 22 total base-pairs;

wherein A represents the sense strand and B1B2 represents the antisense strand;

wherein A is between about 19 nucleotides and about 23 nucleotides;

wherein B1 and B2 are each, individually, between about 1 nucleotide and about 15 nucleotides; and

wherein the combined length of B1+B2 is between about 13 nucleotides and about 23 nucleotides; and

(b) a peptide comprising about 5 to about 40 amino acids,

wherein said siRNA molecule is conjugated to said peptide.

52. A composition comprising:

(a) a small inhibitory nucleic acid (siRNA) molecule, said siRNA molecule comprising three strands A, B1, and B2 (A:B1B2);

wherein A:B1B2 comprises between about 14 total base-pairs and about 24 total base-pairs;

wherein A represents the antisense strand and B1B2 represents the sense strand;

wherein A is between about 14 nucleotides and about 24 nucleotides;

wherein B1 and B2 are each, individually, between about 1 nucleotide and about 15 nucleotides; and

wherein the combined length of B1+B2 is between about 18 nucleotides and about 24 nucleotides; and

(b) a peptide comprising about 5 to about 40 amino acids,

wherein said siRNA molecule is conjugated to said peptide.

53. A composition comprising:

(a) a small inhibitory nucleic acid (siRNA) molecule, said siRNA molecule comprising three strands A, B1, and B2 (A:B1B2);

wherein A:B1B2 comprises between about 14 total base-pairs and about 22 total base-pairs;

wherein A represents the antisense strand and B1B2 represents the sense strand;

wherein A is between about 16 nucleotides and about 22 nucleotides;

wherein B1 and B2 are each, individually, between about 1 nucleotide and about 15 nucleotides;

wherein the combined length of B1+B2 is between about 18 nucleotides and about 22 nucleotides; and

(b) a peptide comprising about 5 to about 40 amino acids,

wherein said siRNA molecule is conjugated to said peptide.

54. A composition comprising:

(a) an siRNA molecule of any one of claims 21 and 50-53 wherein said siRNA molecule is effective in reducing the titer of a target virus selected from the group consisting of a retrovirus, a respiratory viruses, a human metapneumovirus, a human parainfluenza virus, and an influenza virus; and

(b) a peptide comprising about 5 to about 40 amino acids,

wherein said siRNA molecule is conjugated to said peptide.

55. The composition of any one of claims 21, and 50-53 wherein said peptide comprises an amino acid sequence selected from the group consisting of KRRQRRR (SEQ ID NO: 1), RQIKIWFQNRRMKWKK (SEQ ID NO: 2), DAATATRGRSAASRPTERPRAPARSASRPRRPVD (SEQ ID NO: 3), AAVALLPAVLLALLAP (SEQ ID NO: 4), AAVLLPVLLPVLLAAP (SEQ ID NO: 5), VTVLALGALAGVGVG (SEQ ID NO: 6), GALFLGWLGAAGSTMGA (SEQ ID NO: 7), MGLGLHLLVLAAALQGA (SEQ ID NO: 8), LGTYTQDFNKFHTFPQTAIGVGAP (SEQ ID NO: 9), GWTLNSAGYLLKINLKALAALAKKIL (SEQ ID NO: 10), TPPKKKRKVEDPKKKK (SEQ ID NO: 11), RRRRRRR (SEQ ID NO: 12), KLALKLALKALKAALKLA (SEQ ID NO: 13), GLFGAIAGFIENGWEG (SEQ ID NO: 14), FFGAVIGTIALGVATA (SEQ ID NO: 15), FLGFLLGVGSAIASGV (SEQ ID NO: 16), GVFVLGFLGFLATAGS (SEQ ID NO: 17), GAAIGLAWIPYFGPAA (SEQ ID NO: 18), ACTCPYCKDSEGRGSGDPGKKKQHICHIQGCGKVYGKTSHLRAHLRWHTGERPFMC (SEQ ID NO: 19), ACTCPNCKDGEKRSGEQGKKKHVCHIPDCGKTFRKTSLLRAHVRLHTGERPFVC (SEQ ID NO: 20), ACTCPNCKEGGGRGTNLGKKKQHICHIPGCGKVYGKTSHLRAHLRWHSGERPFVC (SEQ ID NO: 21), ACSCPNCREGEGRGSNEPGKKKQHICHIEGCGKVYGKTSHLRAHLRWHTGERPFIC (SEQ ID NO: 22), RCTCPNCTNEMSGLPPIVGPDERGRKQHICHIPGCERLYGKASHLKTHLRWHTGERPFLC (SEQ ID NO: 23), TCDCPNCQEAERLGPAGVHLRKKNIHSCHIPGCGKVYGKTSHLKAHLRWHTGERPFVC (SEQ ID NO: 24), RCTCPNCKAIKHGDRGSQHTHLCSVPGCGKTYKKTSHLRAHLRKHTGDRPFVC (SEQ ID NO: 25), PQISLKKKIFFFIFSNFRGDGKSRIHICHLCNKTYGKTSHLRAHLRGHAGNKPFAC (SEQ ID NO: 26), WWETWKPFQCRICMRNFSTRQARRNHRRRHR (SEQ ID NO: 27), GKINLKALAALAKKIL (SEQ ID NO: 28), RVIRVWFQNKRCKDKK (SEQ ID NO: 29), GRKKRRQRRRPPQGRKKRRQRRRPPQGRKKRRQRRRPPQ (SEQ ID NO: 30), GEQIAQLIAGYIDIILKKKKSK (SEQ ID NO: 31),

KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 33), KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 37), RKESYSVYVYKVLKQ (SEQ ID NO: 41), KKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 42), VTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 43), AQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 44), KDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 45), KKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 46), KRSRKESYSVYVYKVLKQ (SEQ ID NO: 47), SYSVYVYKVLKQ (SEQ ID NO: 48), VYVYKVLKQ (SEQ ID NO: 49), YKVLKQ (SEQ ID NO: 50), KVLKQ (SEQ ID NO: 51), and KGSKKAVTKAQKKEGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 52).

56. A method for reducing the titer of a target virus, said method comprising the steps of:

(a) selecting a target gene for siRNA-mediated gene silencing wherein said target gene is a target viral gene;

(b) designing and/or synthesizing a suitable siRNA molecule(s) for siRNA mediated gene silencing of the target viral gene, wherein each of said siRNA molecule(s) comprises a gapped or nicked duplex and wherein the gap or nick appears in either a sense strand or in an anti-sense strand of said siRNA duplex;

(c) conjugating said siRNA to a peptide comprising about 5 to about 40 amino acids; and

(d) administering said siRNA molecule(s) to a cell expressing said target viral gene,

wherein the siRNA molecule peptide conjugate is capable of specifically binding to the corresponding target viral mRNA thereby reducing its expression level in the cell.

57. A method for reducing the expression of an endogenous gene, said method comprising the steps of:

(a) selecting a target gene for siRNA-mediated gene silencing wherein said target gene is an endogenous gene;

(b) designing and/or synthesizing a suitable gapped or nicked duplex siRNA molecule(s) for siRNA mediated gene silencing of said endogenous target gene wherein said siRNA molecule comprises a gapped or nicked duplex and wherein the gap or nick appears in either the sense strand or in the anti-sense strand of said siRNA molecule; and

(c) conjugating said siRNA to a peptide comprising about 5 to about 40 amino acids; and

(d) administering the siRNA molecule to a cell expressing the endogenous target gene,

wherein said siRNA molecule peptide conjugate is capable of specifically binding to the corresponding endogenous target mRNA thereby reducing its expression level in the cell.

58. The composition of any one of claims 1-13 for use as a medicament for ameliorating inflammation associated with TNF-α.

59. The composition of claim 58 wherein the inflammation occurs in arthritis.

60. The composition of claim 58 wherein the inflammation occurs in psoriasis.

61. The composition of claim 1 for use as a medicament for ameliorating infection associated with influenza virus.

62. Use of a composition of any one of claims 1-13 in the manufacture of a medicament for ameliorating inflammation associated with TNF-α.

63. The use of claim 62 wherein the inflammation occurs in arthritis.

64. The use of claim 62 wherein the inflammation occurs in psoriasis.

65. Use of a composition of claim 1 in the manufacture of a medicament for ameliorating infection associated with influenza virus.

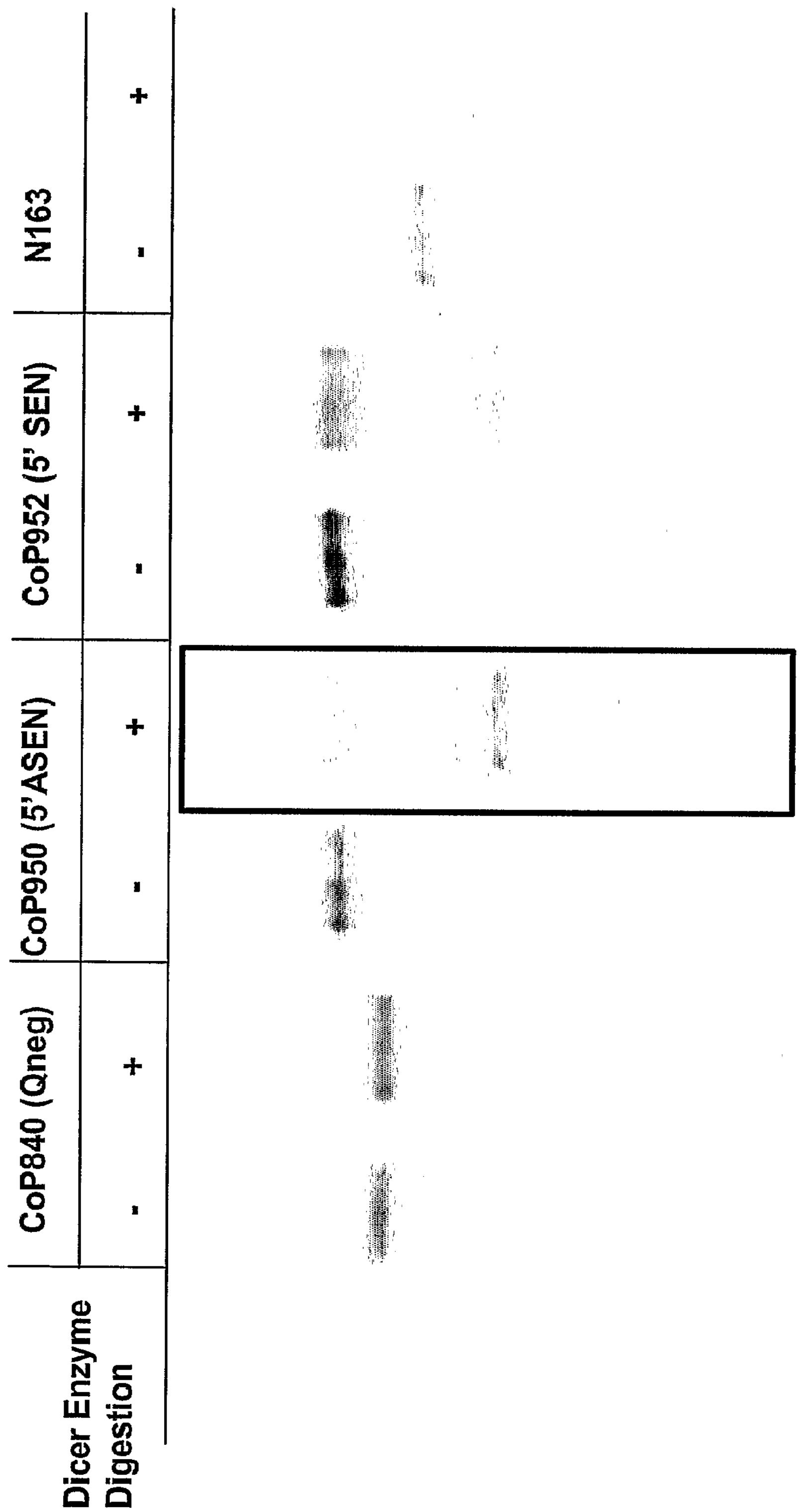

Dicer Enzyme Digestion
CoP840 (Qneg)
CoP950 (5'ASEN)
CoP952 (5' SEN)
N163
-
+
-
+
-
+
-
+

FIG.1

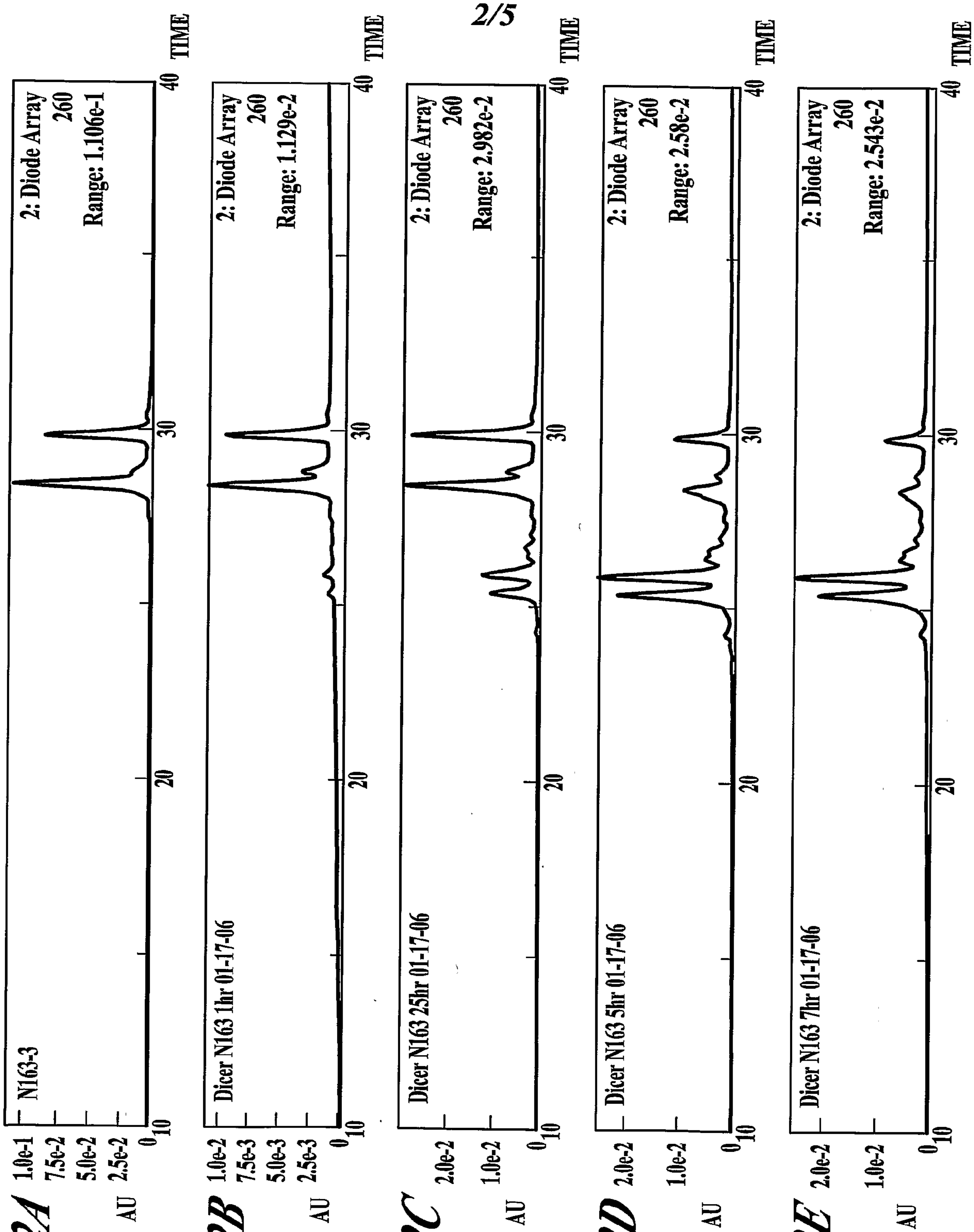


FIG.2A FIG.2B FIG.2C FIG.2D FIG.2E

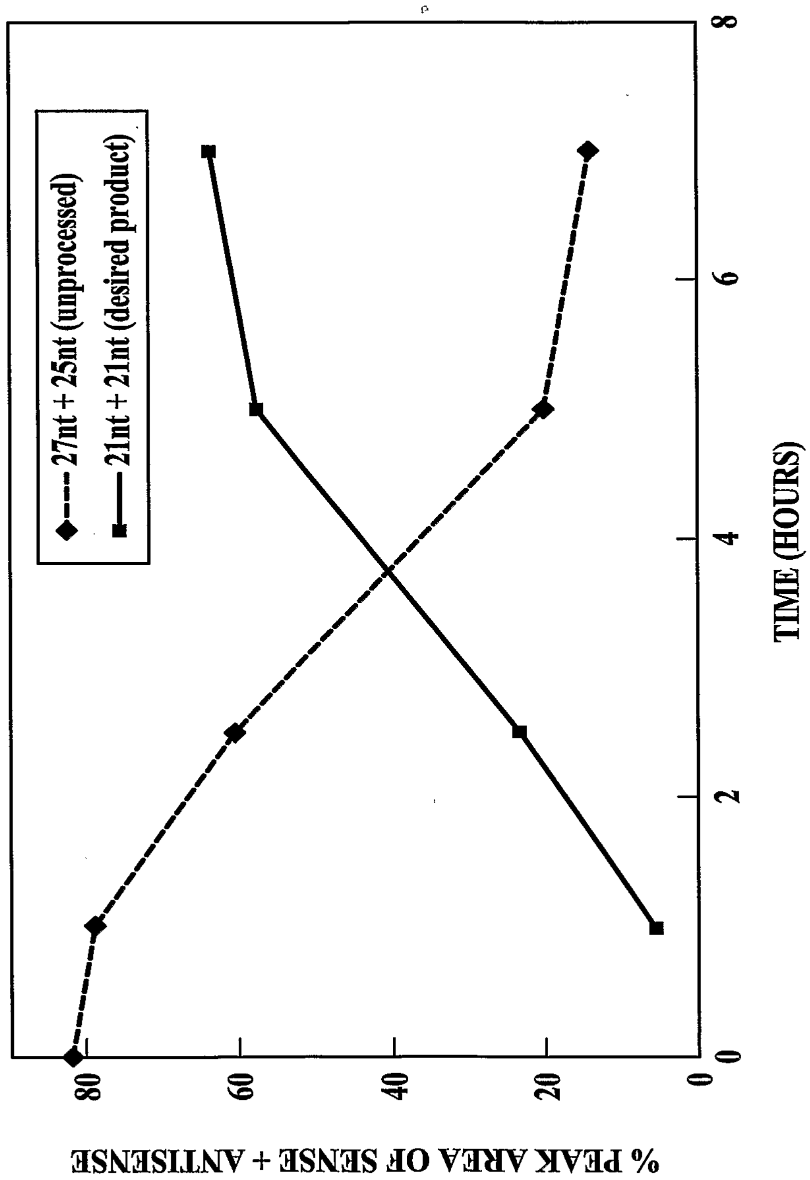

27nt + 25nt (unprocessed)
21nt + 21nt (desired product)
% PEAK AREA OF SENSE + ANTISENSE
0
20
40
60
80
TIME (HOURS)
0
2
4
6
8

FIG.3

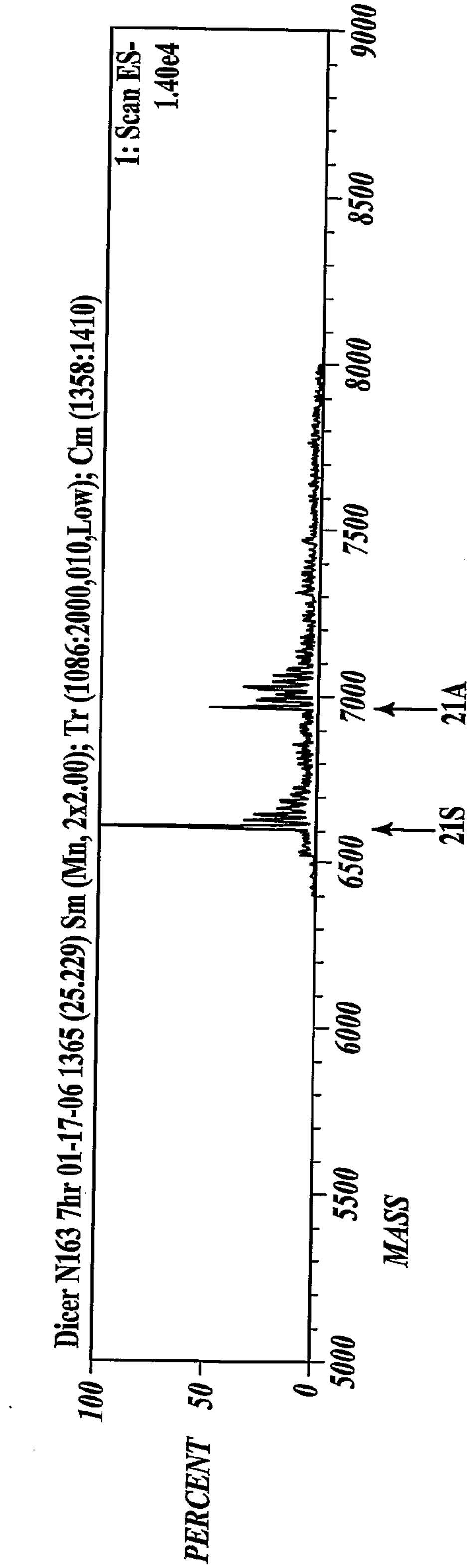

Dicer N163 7hr 01-17-06 1365 (25.229) Sm (Mn, 2x2.00); Tr (1086:2000,010,Low); Cm (1358:1410)
1: Scan ES-
1.40e4
PERCENT
100
50
0
5000
5500
6000
6500
7000
7500
8000
8500
9000
MASS
21S
6606.6
21A
6965.7

FIG.4

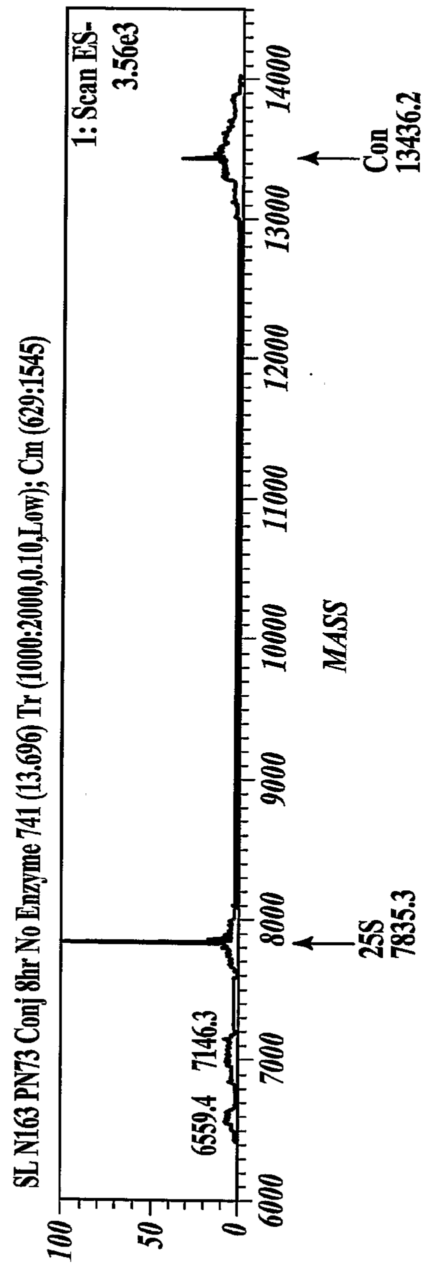

SL N163 PN73 Conj 8hr No Enzyme 741 (13.696) Tr (1000:2000,0.10,Low); Cm (629:1545)
1: Scan ES-
3.56e3
PERCENT
100
50
0
6000
7000
8000
9000
10000
11000
12000
13000
14000
MASS
6559.4
7146.3
25S
7835.3
Con
13436.2

FIG.5A

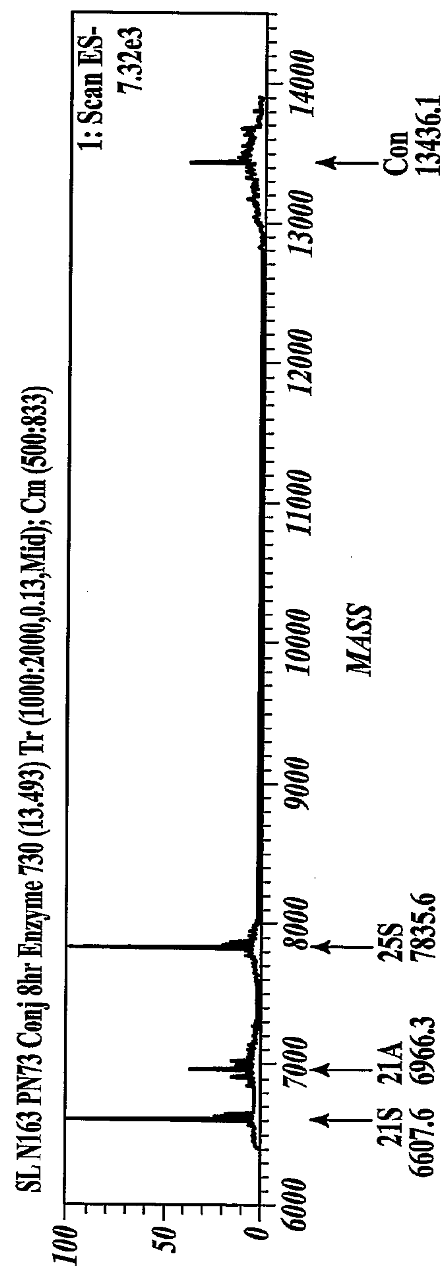

SL N163 PN73 Conj 8hr Enzyme 730 (13.493) Tr (1000:2000,0.13,Mid); Cm (500:833)
1: Scan ES-
7.32e3
PERCENT
100
50
0
6000
7000
8000
9000
10000
11000
12000
13000
14000
MASS
21S
6607.6
21A
6966.3
25S
7835.6
Con
13436.1

FIG.5B

SEQUENCE LISTING

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<110> NASTECH PHARMACEUTICAL COMPANY INC.

<120> PEPTIDE-DICER SUBSTRATE RNA CONJUGATES AS DELIVERY
      VEHICLES FOR SIRNA

<130> 05-20PCT

<140>
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<150> 60/733,665
<151> 2005-04-08

<150> 60/822,896
<151> 2006-08-18

<160> 166

<170> PatentIn Ver. 3.3

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<210> 2
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<212> PRT
<213> Artificial Sequence

<220>
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      peptide

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<212> PRT
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      peptide
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Glu Arg Pro Arg Ala Pro Ala Arg Ser Ala Ser Arg Pro Arg Arg Pro
20 25 30

Val Asp

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peptide

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peptide

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1 5 10 15

<210> 6
<211> 15
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<220>
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peptide

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Val Thr Val Leu Ala Leu Gly Ala Leu Ala Gly Val Gly Val Gly
1 5 10 15

<210> 7
<211> 17
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<220>
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peptide

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Ala

<210> 8
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<213> Artificial Sequence

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1 5 10 15

Ala

<210> 9
<211> 24
<212> PRT
<213> Artificial Sequence

<220>
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peptide

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Thr Ala Ile Gly Val Gly Ala Pro
20

<210> 10
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<213> Artificial Sequence

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peptide

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1 5 10 15

Ala Leu Ala Ala Leu Ala Lys Lys Ile Leu
20 25

<210> 11
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<212> PRT
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<220>
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1 5 10 15

<210> 12
<211> 7
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<220>
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peptide

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1 5

<210> 13
<211> 18
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1 5 10 15

Leu Ala

<210> 14
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<211> 16
<212> PRT
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peptide

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1 5 10 15

<210> 17
<211> 16
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<211> 16
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<210> 19
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1 5 10 15

Asp Pro Gly Lys Lys Lys Gln His Ile Cys His Ile Gln Gly Cys Gly
20 25 30

Lys Val Tyr Gly Lys Thr Ser His Leu Arg Ala His Leu Arg Trp His
35 40 45

Thr Gly Glu Arg Pro Phe Met Cys
50 55

<210> 20
<211> 54
<212> PRT
<213> Artificial Sequence

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peptide

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1 5 10 15

Gln Gly Lys Lys Lys His Val Cys His Ile Pro Asp Cys Gly Lys Thr
20 25 30

Phe Arg Lys Thr Ser Leu Leu Arg Ala His Val Arg Leu His Thr Gly
35 40 45

Glu Arg Pro Phe Val Cys
50

<210> 21
<211> 55
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

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1 5 10 15

Leu Gly Lys Lys Lys Gln His Ile Cys His Ile Pro Gly Cys Gly Lys
20 25 30

Val Tyr Gly Lys Thr Ser His Leu Arg Ala His Leu Arg Trp His Ser
35 40 45

Gly Glu Arg Pro Phe Val Cys
50 55

<210> 22
<211> 56
<212> PRT
<213> Artificial Sequence

<220>
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peptide

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1 5 10 15

Glu Pro Gly Lys Lys Lys Gln His Ile Cys His Ile Glu Gly Cys Gly
20 25 30

Lys Val Tyr Gly Lys Thr Ser His Leu Arg Ala His Leu Arg Trp His
35 40 45

Thr Gly Glu Arg Pro Phe Ile Cys
50 55

<210> 23
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<212> PRT
<213> Artificial Sequence

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peptide

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1 5 10 15

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Ile Val Gly Pro Asp Glu Arg Gly Arg Lys Gln His Ile Cys His Ile
            20                  25                  30

Pro Gly Cys Glu Arg Leu Tyr Gly Lys Ala Ser His Leu Lys Thr His
        35                  40                  45

Leu Arg Trp His Thr Gly Glu Arg Pro Phe Leu Cys
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<210> 24
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<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      peptide

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  1               5                  10                  15

Gly Val His Leu Arg Lys Lys Asn Ile His Ser Cys His Ile Pro Gly
            20                  25                  30

Cys Gly Lys Val Tyr Gly Lys Thr Ser His Leu Lys Ala His Leu Arg
        35                  40                  45

Trp His Thr Gly Glu Arg Pro Phe Val Cys
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<210> 25
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      peptide

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Arg Cys Thr Cys Pro Asn Cys Lys Ala Ile Lys His Gly Asp Arg Gly
  1               5                  10                  15

Ser Gln His Thr His Leu Cys Ser Val Pro Gly Cys Gly Lys Thr Tyr
            20                  25                  30

Lys Lys Thr Ser His Leu Arg Ala His Leu Arg Lys His Thr Gly Asp
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Arg Pro Phe Val Cys
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<212> PRT
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1 5 10 15

Phe Arg Gly Asp Gly Lys Ser Arg Ile His Ile Cys His Leu Cys Asn
20 25 30

Lys Thr Tyr Gly Lys Thr Ser His Leu Arg Ala His Leu Arg Gly His
35 40 45

Ala Gly Asn Lys Pro Phe Ala Cys
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<210> 27
<211> 31
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<213> Artificial Sequence

<220>
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Trp Trp Glu Thr Trp Lys Pro Phe Gln Cys Arg Ile Cys Met Arg Asn
1 5 10 15

Phe Ser Thr Arg Gln Ala Arg Arg Asn His Arg Arg Arg His Arg
20 25 30

<210> 28
<211> 16
<212> PRT
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1 5 10 15

<210> 29
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<210> 30
<211> 39
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<220>
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peptide

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Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Arg Lys Lys Arg Arg
20 25 30

Gln Arg Arg Arg Pro Pro Gln
35

<210> 31
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1 5 10 15

Lys Lys Lys Lys Ser Lys
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<210> 32
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Xaa Xaa His Xaa Xaa Xaa His
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<220>
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20 25 30

Val Leu Lys Gln
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<210> 34
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<400> 35
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<210> 36
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<220>
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Synthetic oligonucleotide

<220>
<223> Description of Artificial Sequence: Synthetic
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<400> 36
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<210> 37
<211> 36
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 37
Lys Gly Ser Lys Lys Ala Val Thr Lys Ala Gln Lys Lys Asp Gly Lys
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Lys Arg Lys Arg Ser Arg Lys Glu Ser Tyr Ser Val Tyr Val Tyr Lys
20 25 30

Val Leu Lys Gln
35

<210> 38
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
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<400> 38
ggatcttatt tcttcggagt t 21

<210> 39
<211> 10
<212> DNA
<213> Artificial Sequence

<220>
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<400> 39
ctccgaagaa 10

<210> 40
<211> 11
<212> DNA
<213> Artificial Sequence

<220>
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<400> 40
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<210> 41
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
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peptide

<400> 41
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1 5 10 15

<210> 42
<211> 33
<212> PRT
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<220>
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1 5 10 15

Arg Ser Arg Lys Glu Ser Tyr Ser Val Tyr Val Tyr Lys Val Leu Lys
20 25 30

Gln

<210> 43
<211> 30
<212> PRT
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<400> 43
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1 5 10 15

Lys Glu Ser Tyr Ser Val Tyr Val Tyr Lys Val Leu Lys Gln
20 25 30

<210> 44
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<220>
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<400> 44
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Tyr Ser Val Tyr Val Tyr Lys Val Leu Lys Gln
20 25

<210> 45
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<220>
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<400> 45
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Tyr Val Tyr Lys Val Leu Lys Gln
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<210> 46
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<220>
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Lys Val Leu Lys Gln
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<220>
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<400> 47
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1 5 10 15

Lys Gln

<210> 48
<211> 12
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<210> 49
<211> 9
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Val Tyr Val Tyr Lys Val Leu Lys Gln
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<210> 50
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<210> 51
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      peptide

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  1   5

<210> 52
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  1   5   10   15

Lys Arg Lys Arg Ser Arg Lys Glu Ser Tyr Ser Val Tyr Val Tyr Lys
             20   25   30

Val Leu Lys Gln
         35

<210> 53
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<220>
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<210> 54
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<400> 54
gaguagauga gguacaggcu u 21

<210> 55
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<210> 56
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<210> 57
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<210> 58
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<210> 59
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Synthetic oligonucleotide

<220>
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cccaugugcu ccucacccac accat 25

<210> 60
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<212> RNA
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<220>
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<400> 60
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<210> 61
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<212> DNA
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Synthetic oligonucleotide

<220>
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oligonucleotide

<400> 61
accucaucua cucccagguc cuctt 25

<210> 62
<211> 27
<212> RNA
<213> Artificial Sequence

<220>
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oligonucleotide

<400> 62
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<210> 63
<211> 27
<212> RNA
<213> Artificial Sequence

<220>
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oligonucleotide

<400> 63
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<210> 64
<211> 29
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<400> 64
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<210> 65
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<212> RNA
<213> Artificial Sequence

<220>
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<210> 66
<211> 27
<212> DNA
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<220>
<223> Description of Combined DNA/RNA Molecule:
Synthetic oligonucleotide

<220>
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oligonucleotide

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<210> 67
<211> 25
<212> RNA
<213> Artificial Sequence

<220>
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oligonucleotide

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<210> 68
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<212> DNA
<213> Artificial Sequence

<220>
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Synthetic oligonucleotide

<220>
<223> Description of Artificial Sequence: Synthetic
oligonucleotide

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<210> 69
<211> 25
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
oligonucleotide

<400> 69
gaucuguucc accauugaag aacuc 25

<210> 70
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Combined DNA/RNA Molecule:
Synthetic oligonucleotide

<220>
<223> Description of Artificial Sequence: Synthetic
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(71) Applicant (*for all designated States except US*): NASTECH PHARMACEUTICAL COMPANY INC. [US/US]; 3830 Monte Villa Parkway, Bothell, WA 98021-7266 (US).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): QUAY, Steven, C. [US/US]; PMB 421, 14241 N.E., Woodinville-Duvall Road,, Woodinville, WA 98072 (US). JOHNSON, Paul, Hickok [US/US]; 12020 211th Place Southeast, Snohomish, WA 98296 (US). HOUSTON, Michael, E., Jr. [CA/US]; 2818 220 Place Northeast, Sammamish, WA 98074 (US). CUI, Kunyuan [US/US]; 3224-189th Street Southeast, Bothell, WA 98012 (US). AHMADIAN, Mohammad [IR/US]; 3106 200th Place Southeast, Bothell, WA 98012 (US). CHEN, Lishan [US/US]; 13620 Southeast 43rd Place, Bellevue, WA 98006 (US). CHEN, Yuching [US/US]; 13620 Southeast 43rd Place, Bellevue, WA 98006 (US). MAYER, Sasha, J. [CA/US]; 6011 174th Street Southeast, Snohomish, WA 98296 (US). FAM, Renata [PL/US]; 1901 Northeast 85th Street, Seattle, WA 98115 (US).

(74) Agents: KNUDSEN, Peter, J. et al.; Nastech Pharmaceutical Company Inc., 3830 Monte Villa Parkway, Bothell, WA 98021-7266 (US).

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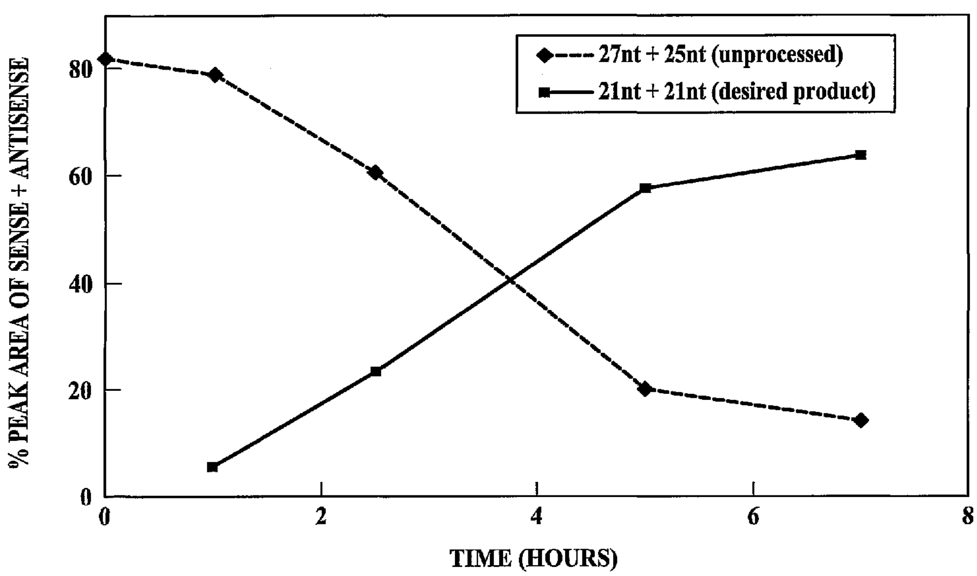
(54) Title: PEPTIDE-DICER SUBSTRATE RNA CONJUGATES AS DELIVERY VEHICLES FOR SIRNA



(57) Abstract: Provided are compositions comprising a double stranded ribonucleic acid (dsRNA) molecule and a peptide of about 5 to about 40 amino acids, wherein the dsRNA molecule is conjugated to the peptide. The strands of the dsRNA may have lengths from about 25 to about 30 base pairs, which may be the same or different. siRNA may, alternatively, comprise at least three strands (i.e., either at least two sense strands and one antisense strand or at least two antisense strands and one sense strand) wherein the at least two sense strands or the at least two antisense strands are separated by a nick or a gap of at least one nucleotide.

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— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

— *of inventorship (Rule 4.17(iv))*

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— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2006/042978

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/48 C12N15/11
ADD. A61P37/00 A61P31/14 A61P31/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03/070897 A (RIBOZYME PHARM INC [US]; MCSWIGGEN JAMES [US]; BEIGELMAN LEONID [US]) 28 August 2003 (2003-08-28) the whole document	3,14-19, 58-60, 62-64
X	WO 2004/028471 A (MASSACHUSETTS INST TECHNOLOGY [US]; CHEN JIANZHU [US]; EISEN HERMAN N) 8 April 2004 (2004-04-08) the whole document	4,5,20, 56,61,65
Y	WO 2004/048545 A (UNIV MASSACHUSETTS [US]; RANA TARIQ M [US]) 10 June 2004 (2004-06-10) figures 3-5; examples 3,4,8,9	1,2, 6-13, 21-55,57

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 July 2007	16/07/2007

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Romano, Alper

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2006/042978

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 03/086273 A2 (YISSUM RES DEV CO [IL]; YEDA RES & DEV [IL]; GILON CHAIM [IL]; LOYTER) 23 October 2003 (2003-10-23) pages 20,21,46; claim 1	1,2, 6-13,57
Y	MURATOVSKA A ET AL: "Conjugate for efficient delivery of short interfering RNA (siRNA) into mammalian cells" FEBS LETTERS, ELSEVIER, AMSTERDAM, NL, vol. 558, no. 1-3, 30 January 2004 (2004-01-30), pages 63-68, XP004488270 ISSN: 0014-5793 the whole document	1,2, 6-13,57
Y	CHIU Y-L ET AL: "VISUALIZING A CORRELATON BETWEEN SIRNA LOCALIZATION, CELLULAR UPTAKE, AND RNAI IN LIVING CELLS" CHEMISTRY AND BIOLOGY, CURRENT BIOLOGY, LONDON, GB, vol. 11, no. 8, August 2004 (2004-08), pages 1165-1175, XP008066695 ISSN: 1074-5521 the whole document	1,2, 6-13,57
Y	SARKAR TUMPA ET AL: "Condensation of oligonucleotides assembled into nicked and gapped duplexes: potential structures for oligonucleotide delivery." NUCLEIC ACIDS RESEARCH 2005, vol. 33, no. 1, 2005, pages 143-151, XP002440840 ISSN: 1362-4962 the whole document	21-55
P,X	WO 2005/117991 A (NASTECH PHARM CO [US]; CHEN LISHAN [US]; CUI KUNYUAN [US] NASTECH PHAR) 15 December 2005 (2005-12-15) the whole document	1-3, 6-19, 57-60, 62-64
P,X	WO 2006/037126 A (NASTECH PHARM CO [US]; CUI KUNYUAN [US]; CHEN LISHAN [US]; CHEN YUCHIN) 6 April 2006 (2006-04-06) the whole document	1-3, 6-19, 57-60, 62-64
P,X	US 2006/008464 A1 (GILON CHAIM [IL] ET AL) 12 January 2006 (2006-01-12) the whole document	1-3, 6-19, 57-60, 62-64

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2006/042978

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-20,56-65

   A composition comprising a dsRNA molecule conjugated to a peptide, methods and uses related thereto

   ---

2. claims: 21-55

   A composition comprising a gapped siRNA molecule conjugated to a peptide

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2006/042978

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03070897 A	28-08-2003	AU 2003217550 A1	09-09-2003
		CA 2477014 A1	28-08-2003
		EP 1478730 A2	24-11-2004
		JP 2005517433 T	16-06-2005
WO 2004028471 A	08-04-2004	AU 2003279004 A1	19-04-2004
		AU 2003279010 A1	19-04-2004
		CA 2500468 A1	08-04-2004
		EP 1658304 A2	24-05-2006
		JP 2006512906 T	20-04-2006
		KR 20050084607 A	26-08-2005
		MX PA05003287 A	05-07-2005
		WO 2004029213 A2	08-04-2004
		US 2006160759 A1	20-07-2006
WO 2004048545 A	10-06-2004	AU 2003298724 A1	18-06-2004
		CA 2506714 A1	10-06-2004
		EP 1585756 A2	19-10-2005
		US 2004204377 A1	14-10-2004
WO 03086273 A2	23-10-2003	AU 2003214625 A1	27-10-2003
WO 2005117991 A	15-12-2005	CA 2565685 A1	15-12-2005
		EP 1750775 A2	14-02-2007
		US 2006035815 A1	16-02-2006
WO 2006037126 A	06-04-2006	AU 2005290336 A1	06-04-2006
		CA 2580996 A1	06-04-2006
		EP 1793864 A2	13-06-2007
US 2006008464 A1	12-01-2006	NONE	

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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60/822,896 18 August 2006 (18.08.2006) US

(71) Applicant *(for all designated States except US)*: **NASTECH PHARMACEUTICAL COMPANY INC.** [US/US]; 3830 Monte Villa Parkway, Bothell, WA 98021-7266 (US).

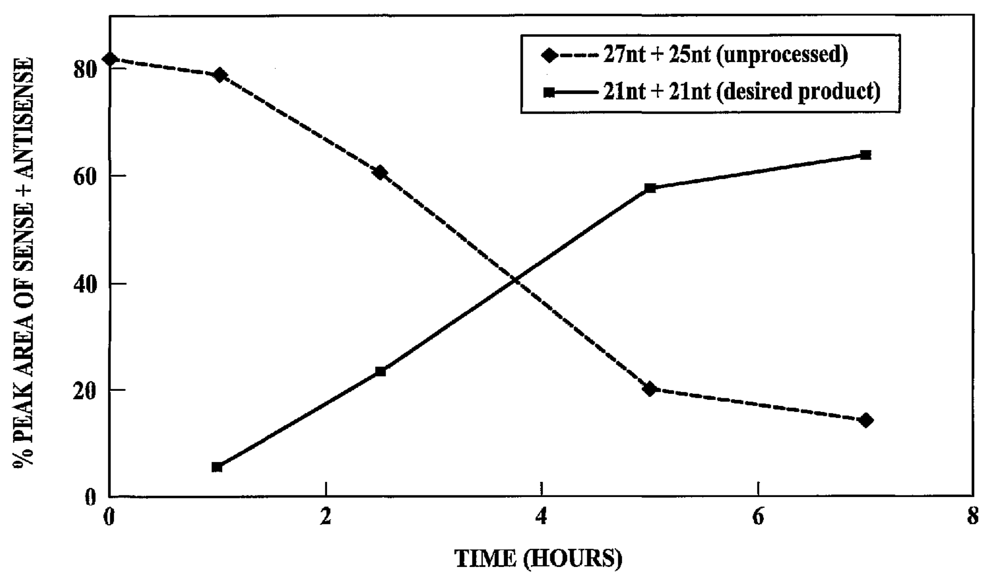
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **QUAY, Steven, C.** [US/US]; PMB 421, 14241 N.E., Woodinville-Duvall Road,, Woodinville, WA 98072 (US). **JOHNSON, Paul, Hickok** [US/US]; 12020 211th Place Southeast, Snohomish, WA 98296 (US). **HOUSTON, Michael, E., Jr.** [CA/US]; 2818 220 Place Northeast, Sammamish, WA 98074 (US). **CUI, Kunyuan** [US/US]; 3224-189th Street Southeast, Bothell, WA 98012 (US). **AHMADIAN, Mohammad** [IR/US]; 3106 200th Place Southeast, Bothell, WA 98012 (US). **CHEN, Lishan** [US/US]; 13620 Southeast 43rd Place, Bellevue, WA 98006 (US). **CHEN, Yuching** [US/US]; 13620 Southeast 43rd Place, Bellevue, WA 98006 (US). **MAYER, Sasha, J.** [CA/US]; 6011 174th Street Southeast, Snohomish, WA 98296 (US). **FAM, Renata** [PL/US]; 1901 Northeast 85th Street, Seattle, WA 98115 (US).

(74) Agents: **KNUDSEN, Peter, J.** et al.; Nastech Pharmaceutical Company Inc., 3830 Monte Villa Parkway, Bothell, WA 98021-7266 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS,

[Continued on next page]

(54) Title: PEPTIDE-DICER SUBSTRATE RNA CONJUGATES AS DELIVERY VEHICLES FOR SIRNA



(57) Abstract: Provided are compositions comprising a double stranded ribonucleic acid (dsRNA) molecule and a peptide of about 5 to about 40 amino acids, wherein the dsRNA molecule is conjugated to the peptide. The strands of the dsRNA may have lengths from about 25 to about 30 base pairs, which may be the same or different. siRNA may, alternatively, comprise at least three strands (i.e., either at least two sense strands and one antisense strand or at least two antisense strands and one sense strand) wherein the at least two sense strands or the at least two antisense strands are separated by a nick or a gap of at least one nucleotide.

WO 2007/056153 A3

RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
- *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*
- *of inventorship (Rule 4.17(iv))*

Published:
- *with international search report*
- *with amended claims*
- *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

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Date of publication of the amended claims: 25 October 2007

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

AMENDED CLAIMS
[received by the International Bureau on 14 September 2007 (14.09.2007)]

CLAIMS

1. A composition comprising:

(a) a double stranded ribonucleic acid (dsRNA) molecule, wherein the strands have lengths from about 25 to about 30 nucleotides which may be the same or different; and

(b) a peptide comprising about 5 to about 40 amino acids, wherein the peptide contains the amino acid sequence KVLKQ (SEQ ID NO: 51);

wherein the dsRNA molecule is conjugated to the peptide.

2. The composition of claim 1, wherein the dsRNA molecule is an siRNA.

3. The composition of claim 2, wherein the siRNA contains a nucleic sequence homologous to a portion of the nucleic sequence of a human TNF-alpha gene.

4. The composition of claim 2, wherein the siRNA contains a nucleic sequence homologous to a portion of the nucleic sequence of a viral gene.

5. The composition of claim 4, wherein the source of the viral gene is an influenza virus.

6. The composition of claim 1, further comprising a carrier.

7. The composition of claim 1, wherein the dsRNA molecule further comprises a single stranded 3' antisense strand overhang comprising 2 nucleotides.

8. The composition of claim 1, wherein the dsRNA molecule further comprises a single stranded 3' sense strand overhang comprising 2 nucleotides.

9. The composition of claim 1, wherein the dsRNA molecule has no overhang.

10. The composition of claim 1, wherein the strands have lengths from about 25 to about 29 nucleotides which may be the same or different.

11. The composition of claim 1, wherein the dsRNA molecule consists of a sense RNA strand and an antisense RNA strand, and the peptide is conjugated to the 5' end of the antisense RNA strand.

12. The composition of claim 1, wherein the amino acid sequence of the peptide is selected from the group consisting of:
KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 33);
KKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 42);
VTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 43);
AQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 44);
KDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 45);
KKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 46);
KRSRKESYSVYVYKVLKQ (SEQ ID NO: 47);
RKESYSVYVYKVLKQ (SEQ ID NO: 41); SYSVYVYKVLKQ (SEQ ID NO: 48);
VYVYKVLKQ (SEQ ID NO: 49); YKVLKQ (SEQ ID NO: 50); and
KVLKQ (SEQ ID NO: 51).

13. The composition of claim 1, wherein the peptide is conjugated to a molecule that binds to a cell in an animal.

14. Use of the composition as in any one of claims 1-3 or 6-13 for ameliorating inflammation associated with TNF-α comprising administering an ameliorating amount of the composition to an animal.

15. Use of the composition as in any one of claims 1-3 or 6-13 in the manufacture of a medicament for ameliorating inflammation associated with TNF-α in an animal.

16. The use of claims 14 or 15, wherein the inflammation occurs in arthritis.

17. The use of claims 14 or 15, wherein the inflammation occurs in psoriasis.

18. Use of a pharmaceutical composition for inhibiting expression of a gene in an animal for ameliorating inflammation comprising administering a double stranded ribonucleic acid (dsRNA) molecule to the animal, wherein the pharmaceutical

composition comprises the dsRNA molecule and a peptide, wherein the dsRNA molecule comprises about 25 to about 30 base pairs, wherein the peptide comprises about 5 to about 40 amino acids and comprises the amino acid sequence KVLKQ (SEQ ID NO: 51), and wherein the dsRNA molecule is conjugated to the peptide.

19. Use of a pharmaceutical composition comprising a double stranded ribonucleic acid (dsRNA) molecule and a peptide in the manufacture of a medicament for inhibiting expression of a gene in an animal for ameliorating inflammation , wherein the dsRNA molecule comprises about 25 to about 30 base pairs, wherein the peptide comprises about 5 to about 40 amino acids and comprises the amino acid sequence KVLKQ (SEQ ID NO: 51), and wherein the dsRNA molecule is conjugated to the peptide.

20. The use of claims 18 or 19, wherein the inflammation occurs in arthritis.

21. The use of claims 18 or 19, wherein the inflammation occurs in psoriasis.

22. Use of the composition of claim 1 for ameliorating infection associated with influenza virus comprising administering an ameliorating amount of the composition to an animal.

23. Use of the composition of claim 1 in the manufacture of a medicament for ameliorating infection associated with influenza virus in an animal.

24. A composition comprising:

(a) a small inhibitory nucleic acid (siRNA) molecule,

the siRNA molecule comprising a first RNA strand (A strand) of between about 15 nucleotides and about 50 nucleotides, a second RNA strand (B1 strand) of between about between about 1 nucleotide and about 25 nucleotides, and a third RNA strand (B2 strand) of between about 1 nucleotide and about 25 nucleotides;

wherein the dsRNA molecule is conjugated to the peptide.

wherein the B1 strand and the B2 strand are each complementary to non-overlapping regions of the A strand;

wherein a first double-stranded region (A:B1) is formed by annealing the B1 strand and the A strand; and

wherein a second double-stranded region (A:B2) is formed by annealing the B2 strand and the A strand; and

(b) a peptide comprising about 5 to about 40 amino acids,

wherein the siRNA molecule is conjugated to the peptide.

25. The composition of claim 24 wherein the A:B1 duplex is separated from the A:B2 duplex by a nick or by a gap wherein the gap results from at least one unpaired nucleotide in the A strand that is positioned between the A:B1 duplex and the A:B2 duplex.

26. The composition of claim 25 further comprising one or more unpaired nucleotide(s) at the 3' end of either or both of the A strand, the B1 strand, and/or the B2 strand.

27. The composition of any of claims 25-26 wherein the A strand is between about 18 nucleotides and about 40 nucleotides.

28. The composition of claim 27 wherein the A strand is between about 20 nucleotides and about 32 nucleotides.

29. The composition of claim 28 wherein the A strand is 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or 31 nucleotides.

30. The composition of any one of claims 25-26 wherein the A:B1 duplex and the A:B2 duplex comprise, in sum, between about 15 base-pairs and about 40 base-pairs.

31. The composition of claim 30 wherein the A:B1 duplex and the A:B2 duplex comprise, in sum, between about 18 base-pairs and about 35 base-pairs.

32. The composition of claim 31 wherein the A:B1 duplex and the A:B2 duplex comprise, in sum, between about 20 base-pairs and about 30 base-pairs.

33. The composition of claim 32 wherein the A:B1 duplex and the A:B2 duplex comprise, in sum, either 21, 22, 23, 24, 25, 26, 27, 28, or29 base-pairs.

34. The composition of claim 26 wherein the siRNA molecule comprises one or two single-strand 3' overhang(s) of between 1 nucleotide and 5 nucleotides.

35. The composition of any one of claims 25-26 wherein the A strand comprises the nucleotide sequence 3'TTCCUAGAAUAAAGAAGCCUCUGUUAC5' (SEQ ID NO: 76).

36. The composition of claim 22 wherein the B1 strand comprises the nucleotide sequence 5'GGAUCU3' (SEQ ID NO: 77).

37. The composition of claim 25 wherein the B2 strand comprises the nucleotide sequence 5'ACAAUG3' (SEQ ID NO: 90).

38. The composition of claim 25 wherein the A:B1 duplex is separated from the A:B2 duplex by a nick.

39. The composition of claim 38 wherein the B2 strand terminates with a 5' hydroxyl.

40. The composition of claim 38 wherein the B1 strand comprises the nucleotide sequence 5'GGAUCUUAUUU3' (SEQ ID NO: 135), wherein the B2 strand comprises the nucleotide sequence 5'CUUCGGAGTT3' (SEQ ID NO: 136), and wherein the A strand comprises the nucleotide sequence 5'CUCCGAAGAAAUAAGAUCCTT3' (SEQ ID NO: 137).

41. The composition of claim 38 wherein the B1 strand comprises the nucleotide sequence 5'GGATCTTATTT3' (SEQ ID NO: 144), wherein the B2 strand comprises the nucleotide sequence 5'CTTCGGAGTT3' (SEQ ID NO: 145), and wherein the A strand comprises the nucleotide sequence 5'CTCCGAAGAAATAAGATCCTT3' (SEQ ID NO: 146).

42. The composition of claim 38 wherein the B1 strand comprises the nucleotide sequence 5'CTCCGAAGAA3' (SEQ ID NO: 148), wherein the B2 strand comprises the nucleotide sequence 5'ATAAGATCCTT3' (SEQ ID NO: 149), and wherein the A strand comprises the nucleotide sequence 5'GGATCTTATT TCTTCGGAGTT3' (SEQ ID NO: 147).

43. The composition of claim 38 wherein the B1 strand comprises the nucleotide sequence 5'GGAUCUUAUUU3' (SEQ ID NO: 153), wherein the B2 strand comprises the nucleotide sequence 5'CUUCGGAGTT3' (SEQ ID NO: 154), and wherein the A strand comprises the nucleotide sequence 5'CUCCGAAGAAAUAAGAUCCTT3' (SEQ ID NO: 155).

44. The composition of claim 38 wherein the B1 strand comprises the nucleotide sequence 5'GGATCTTATTT3' (SEQ ID NO: 159), wherein the B2 strand comprises the nucleotide sequence 5'CTTCGGAGTT3' (SEQ ID NO: 160), and wherein the A strand comprises the nucleotide sequence 5'CTCCGAAGAAATAAGATCCTT3' (SEQ ID NO: 161).

45. The composition of claim 38 wherein the B1 strand comprises the nucleotide sequence 5'CTCCGAAGAA3' (SEQ ID NO: 166), wherein the B2 strand comprises the nucleotide sequence 5'ATAAGATCCTT3' (SEQ ID NO: 34), and wherein the A strand comprises the nucleotide sequence 5'GGATCTTATT TCTTCGGAGTT3' (SEQ ID NO: 165).

46. The composition of claim 38 wherein the B1 strand terminates with a 5' phosphate.

47. The composition of claim 46 wherein the B1 strand comprises the nucleotide sequence 5'GGAUCUUAUUU3' (SEQ ID NO: 138), wherein the B2 strand comprises the nucleotide sequence 5'CUUCGGAGTT3' (SEQ ID NO: 139), and wherein the A strand comprises the nucleotide sequence 5'CUCCGAAGAAAUAAGAUCCTT3' (SEQ ID NO: 140).

48. The composition of claim 46 wherein the B1 strand comprises the nucleotide sequence 5'GGATCTTATTT3' (SEQ ID NO: 141), wherein the B2 strand comprises the nucleotide sequence 5'CTTCGGAGTT3' (SEQ ID NO: 142), and wherein the A strand comprises the nucleotide sequence 5'CTCCGAAGAAATAAGATCCTT3' (SEQ ID NO: 143).

49. The composition of claim 46 wherein the B1 strand comprises the nucleotide sequence 5'CTCCGAAGAA3' (SEQ ID NO: 151), wherein the B2 strand comprises the nucleotide sequence 5'ATAAGATCCTT3' (SEQ ID NO: 152), and wherein the A strand comprises the nucleotide sequence 5' GGATCTTATTTCTTCGGAGTT 3' (SEQ ID NO: 150).

50. The composition of claim 46 wherein the B1 strand comprises the nucleotide sequence 5'GGAUCUUAUUU3' (SEQ ID NO: 156), wherein the B2 strand comprises the nucleotide sequence 5'CUUCGGAGTT3' (SEQ ID NO: 157), and wherein the A strand comprises the nucleotide sequence 5'CUCCGAAGAAAUAAGAUCCTT3' (SEQ ID NO: 158).

51. The composition of claim 46 wherein the B1 strand comprises the nucleotide sequence 5'GGATCTTATTT3' (SEQ ID NO: 162), wherein the B2 strand comprises the nucleotide sequence 5'CTTCGGAGTT3' (SEQ ID NO: 163), and wherein the A strand comprises the nucleotide sequence 5'CTCCGAAGAAATAAGATCCTT3' (SEQ ID NO: 164).

52. The composition of claim 46 wherein the B1 strand comprises the nucleotide sequence 5'CTCCGAAGAA3' (SEQ ID NO: 39), wherein the B2 strand comprises the nucleotide sequence 5'ATAAGATCCTT3' (SEQ ID NO: 40), and wherein the A strand comprises the nucleotide sequence 5'GGATCTTATTTCTTCGGAGTT3' (SEQ ID NO: 38).

53. A composition comprising:

(a) a small inhibitory nucleic acid (siRNA) molecule, the siRNA molecule comprising three strands A, B1, and B2 (A:B1B2);

wherein A:B1B2 comprises between about 14 total base-pairs and about 24 total base-pairs;

wherein A represents the sense strand and B1B2 represents the antisense strand;

wherein A is between about 19 nucleotides and about 25 nucleotides;

wherein B1 and B2 are each, individually, between about 1 nucleotide and about 15 nucleotides; and

wherein the combined length of B1 and B2 is between about 13 nucleotides and about 23 nucleotides; and

(b) a peptide comprising about 5 to about 40 amino acids,

wherein the siRNA molecule is conjugated to the peptide.

54. A composition comprising:

(a) a small inhibitory nucleic acid (siRNA) molecule, the siRNA molecule comprising three strands A, B1, and B2 (A:B1B2);

wherein A:B1B2 comprises between about 16 total base-pairs and about 22 total base-pairs;

wherein A represents the sense strand and B1B2 represents the antisense strand;

wherein A is between about 19 nucleotides and about 23 nucleotides;

wherein B1 and B2 are each, individually, between about 1 nucleotide and about 15 nucleotides; and

wherein the combined length of B1 and B2 is between about 13 nucleotides and about 23 nucleotides; and

(b) a peptide comprising about 5 to about 40 amino acids,

wherein the siRNA molecule is conjugated to the peptide.

55. A composition comprising:

(a) a small inhibitory nucleic acid (siRNA) molecule, the siRNA molecule comprising three strands A, B1, and B2 (A:B1B2);

wherein A:B1B2 comprises between about 14 total base-pairs and about 24 total base-pairs;

wherein A represents the antisense strand and B1B2 represents the sense strand;

wherein A is between about 14 nucleotides and about 24 nucleotides;

wherein B1 and B2 are each, individually, between about 1 nucleotide and about 15 nucleotides; and

wherein the combined length of B1 and B2 is between about 18 nucleotides and about 24 nucleotides; and

(b) a peptide comprising about 5 to about 40 amino acids,

wherein the siRNA molecule is conjugated to the peptide.

56. A composition comprising:

(a) a small inhibitory nucleic acid (siRNA) molecule, the siRNA molecule comprising three strands A, B1, and B2 (A:B1B2);

wherein A:B1B2 comprises between about 14 total base-pairs and about 22 total base-pairs;

wherein A represents the antisense strand and B1B2 represents the sense strand;

wherein A is between about 16 nucleotides and about 22 nucleotides;

wherein B1 and B2 are each, individually, between about 1 nucleotide and about 15 nucleotides;

wherein the combined length of B1 and B2 is between about 18 nucleotides and about 22 nucleotides; and

(b) a peptide comprising about 5 to about 40 amino acids,

wherein the siRNA molecule is conjugated to the peptide.

57. A composition comprising:

(a) an siRNA molecule as in any one of claims 24 and 53-56 wherein the siRNA molecule is effective in reducing the titer of a target virus selected from the group consisting of a retrovirus, a respiratory viruses, a human metapneumovirus, a human parainfluenza virus, and an influenza virus; and

(b) a peptide comprising about 5 to about 40 amino acids,

wherein the siRNA molecule is conjugated to the peptide.

58. The composition as in any one of claims 24, and 53-56 wherein the peptide comprises an amino acid sequence selected from the group consisting of KRRQRRR (SEQ ID NO: 1), RQIKIWFQNRRMKWKK (SEQ ID NO: 2), DAATATRGRSAASRPTERPRAPARSASRPRRPVD (SEQ ID NO: 3), AAVALLPAVLLALLAP (SEQ ID NO: 4), AAVLLPVLLPVLLAAP (SEQ ID NO: 5), VTVLALGALAGVGVG (SEQ ID NO: 6), GALFLGWLGAAGSTMGA (SEQ ID NO: 7), MGLGLHLLVLAAALQGA (SEQ ID NO: 8), LGTYTQDFNKFHTFPQTAIGVGAP (SEQ ID NO: 9), GWTLNSAGYLLKINLKALAALAKKIL (SEQ ID NO: 10), TPPKKKRKVEDPKKKK (SEQ ID NO: 11), RRRRRRR (SEQ ID NO: 12), KLALKLALKALKAALKLA (SEQ ID NO: 13), GLFGAIAGFIENGWEG (SEQ ID

NO: 14), FFGAVIGTIALGVATA (SEQ ID NO: 15), FLGFLLGVGSAIASGV (SEQ ID NO: 16), GVFVLGFLGFLATAGS (SEQ ID NO: 17), GAAIGLAWIPYFGPAA (SEQ ID NO: 18),
ACTCPYCKDSEGRGSGDPGKKKQHICHIQGCGKVYGKTSHLRAHLRWHTGERPF MC (SEQ ID NO: 19),
ACTCPNCKDGEKRSGEQGKKKHVCHIPDCGKTFRKTSLLRAHVRLHTGERPFVC (SEQ ID NO: 20),
ACTCPNCKEGGGRGTNLGKKKQHICHIPGCGKVYGKTSHLRAHLRWHSGERPF VC (SEQ ID NO: 21),
ACSCPNCREGEGRGSNEPGKKKQHICHIEGCGKVYGKTSHLRAHLRWHTGERPF IC (SEQ ID NO: 22),
RCTCPNCTNEMSGLPPIVGPDERGRKQHICHIPGCERLYGKASHLKTHLRWHTGE RPFLC (SEQ ID NO: 23),
TCDCPNCQEAERLGPAGVHLRKKNIHSCHIPGCGKVYGKTSHLKAHLRWHTGER PFVC (SEQ ID NO: 24),
RCTCPNCKAIKHGDRGSQHTHLCSVPGCGKTYKKTSHLRAHLRKHTGDRPFVC (SEQ ID NO: 25),
PQISLKKKIFFFIFSNFRGDGKSRIHICHLCNKTYGKTSHLRAHLRGHAGNKPFAC (SEQ ID NO: 26),
WWETWKPFQCRICMRNFSTRQARRNHRRRHR (SEQ ID NO: 27),
GKINLKALAALAKKIL (SEQ ID NO: 28), RVIRVWFQNKRCKDKK (SEQ ID NO: 29), GRKKRRQRRRPPQGRKKRRQRRRPPQGRKKRRQRRRPPQ (SEQ ID NO: 30),
GEQIAQLIAGYIDIILKKKKSK (SEQ ID NO: 31),
KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 33),
KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 37),
RKESYSVYVYKVLKQ (SEQ ID NO: 41),
KKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 42),
VTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 43),
AQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 44),
KDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 45),
KKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 46),

KRSRKESYSVYVYKVLKQ (SEQ ID NO: 47), SYSVYVYKVLKQ (SEQ ID NO: 48), VYVYKVLKQ (SEQ ID NO: 49), YKVLKQ (SEQ ID NO: 50), KVLKQ (SEQ ID NO: 51), and KGSKKAVTKAQKKEGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 52).

59. Use of an siRNA molecule for reducing the titer of a target virus, the use comprising the steps of:

(a) selecting a target gene for siRNA-mediated gene silencing wherein the target gene is a target viral gene;

(b) designing and/or synthesizing a suitable siRNA molecule(s) for siRNA mediated gene silencing of the target viral gene, wherein each of the siRNA molecule(s) comprises a gapped or nicked duplex and wherein the gap or nick appears in either a sense strand or in an anti-sense strand of the siRNA duplex;

(c) conjugating the siRNA to a peptide comprising about 5 to about 40 amino acids; and

(d) administering the siRNA molecule(s) to a cell expressing the target viral gene,

wherein the siRNA molecule peptide conjugate is capable of specifically binding to the corresponding target viral mRNA thereby reducing its expression level in the cell.

60. Use of an siRNA molecule(s) for the manufacture of a medicament for reducing the titer of a target virus by siRNA-mediated gene silencing of a target viral gene, wherein each of the siRNA molecule(s) comprises a gapped or nicked duplex and wherein the gap or nick appears in either a sense strand or in an anti-sense strand of the siRNA duplex; wherein the siRNA is conjugated to a peptide comprising about 5 to about 40 amino acids; and wherein the siRNA molecule peptide conjugate is capable of specifically binding to the corresponding target viral mRNA thereby reducing its expression level in the cell.

61. Use of an siRNA molecule for reducing the expression of an endogenous gene, the use comprising the steps of:

(a) selecting a target gene for siRNA-mediated gene silencing wherein the target gene is an endogenous gene;

(b) designing and/or synthesizing a suitable gapped or nicked duplex siRNA molecule(s) for siRNA mediated gene silencing of the endogenous target gene wherein the siRNA molecule comprises a gapped or nicked duplex and wherein the gap or nick appears in either the sense strand or in the anti-sense strand of the siRNA molecule; and

(c) conjugating the siRNA to a peptide comprising about 5 to about 40 amino acids; and

(d) administering the siRNA molecule to a cell expressing the endogenous target gene,

wherein the siRNA molecule peptide conjugate is capable of specifically binding to the corresponding endogenous target mRNA thereby reducing its expression level in the cell.

62. Use of an siRNA molecule(s) for the manufacture of a medicament for reducing the expression of an endogenous gene by siRNA mediated gene silencing of the endogenous target gene, wherein the siRNA molecule comprises a gapped or nicked duplex and wherein the gap or nick appears in either the sense strand or in the anti-sense strand of the siRNA molecule; and wherein the siRNA is conjugated to a peptide comprising about 5 to about 40 amino acids; and wherein the siRNA molecule peptide conjugate is capable of specifically binding to the corresponding endogenous target mRNA thereby reducing its expression level in the cell.

63. The composition of any of claims 1-13 or 24-58 for use in medicine.

64. The composition in any one of claims 1-3 or 6-13 for use as a medicament for ameliorating inflammation associated with TNF-α.

65. The composition of claim 64 wherein the inflammation occurs in arthritis.

66. The composition of claim 64 wherein the inflammation occurs in psoriasis.

67. The composition of claim 1 for use as a medicament for ameliorating infectionassociated with influenza virus.